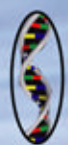




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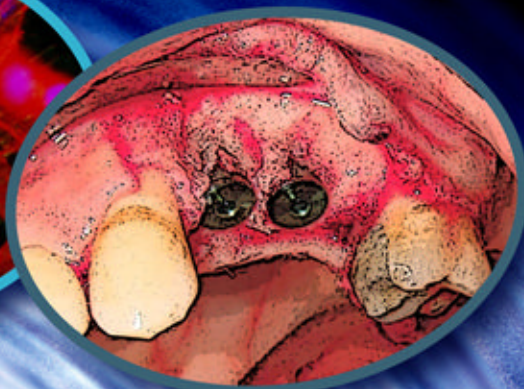
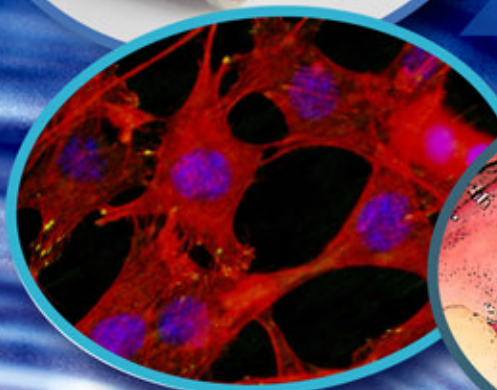


Implant Dentistry Research Guide

Basic, Translational
and Clinical Research

Ahmed Ballo
Editor

*Dental Science, Materials
and Technology*



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DENTAL SCIENCE, MATERIALS AND TECHNOLOGY

IMPLANT DENTISTRY RESEARCH GUIDE

BASIC, TRANSLATIONAL AND CLINICAL RESEARCH

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IMPLANT DENTISTRY RESEARCH GUIDE

BASIC, TRANSLATIONAL AND CLINICAL RESEARCH

AHMED BALLO
EDITOR



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New York

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Preface

Professor Brånemark's discovery of the osseointegration phenomenon in 1965 was an important contribution to implant dentistry. Since then, the rapid development of the field by the introduction of new biomaterials, designs, and techniques has led to an expansion in the clinical indications for this modality of treatment.

Advances in molecular biology and biochemistry, cell biology, developmental biology, wound-healing physiology, materials science and engineering, and novel laboratory techniques, as well as in laboratory and clinical instrumentation, have provided incentives and opportunities for renewed and ever-increasing interest in detailed studies of the mechanisms of osseointegration.

Implant dentistry research tasks and activities are widely diverse and require certain skill sets and educational backgrounds to both plan and execute.

The *Implant Dentistry Research Guide* is an attempt to draw the attention of the dental community toward the need for dentists trained as clinician researchers or scientists, bringing their clinical expertise into the laboratory and translating laboratory innovations to patient care—that is, from “bedside to bench to bedside”—especially in the field of implant dentistry and guided-bone-regeneration research. The *Guide* has 6 parts and is concise but inclusive.

I extend my sincere thanks to all the colleagues who shared this vision and interest and contributed to this textbook. I would like also to thank Mr Mark Hill the (Head of Global Communication at Straumann) who has been supportive of book front cover.

I also greatly appreciate the BIOMATCELL VINN Excellence Center of Biomaterials and Cell Therapy in Gothenburg, Sweden for funding and supporting this textbook.

Finally, I would like to thank my wife, Yasmina, my kids, Mansour and Samiha, for their support and understanding when working on this book required my nights and weekends.

Ahmed M. Ballo, BDS, EDA, PhD
Gothenburg, September 15, 2011

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Dr. Ahmed Ballo obtained his Bachelor of Dental Surgery (BDS) degree from the Faculty of Dentistry of the University of Garyounis in Benghazi, Libya, in 2002. He completed one year Fellowship in Implant Dentistry as a Scholar of the International Team for Implantology (ITI) of the Institute of Dentistry, University of Turku, Turku, Finland, in 2007 and received his Doctor of Philosophy (PhD) degree from the Department of Prosthetic Dentistry and Biomaterials of the Institute of Dentistry, University of Turku. For his doctoral work, Dr. Ballo received a Certificate of Honor in Recognition of a Thesis Book of Exceptional Merit from the University of Turku in 2008 and the European Biomaterials and Tissue Engineering Doctoral Award (EDA) from the European Society for Biomaterials (ESB), Lausanne, Switzerland, in 2009.

Dr. Ballo joined the Department of Biomaterials at Sahlgrenska Academe of the Institute of Clinical Science, University of Gothenburg, Gothenburg, Sweden, as a Postdoctoral Associate in 2008 and worked in the BIOMATCELL Center of Biomaterials and Cell Therapy. He was actively involved in teaching undergraduate and postgraduate students in courses arranged by the Department of Biomaterials at the University of Gothenburg. He has also established a postgraduate course entitled “Models and Methods in Dental Implant Research” at the same department. After his postdoctoral training, Dr. Ballo moved to

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Dr. Ballo participates as an invited lecturer in national and international conferences and congresses. Recently, he has become a consultant of the Dental Implant and Osseointegration Research Chair in the College of Dentistry at King Saud University, Riyadh, Saudi Arabia.

Dr. Ballo's current research interests include applied and clinical research in osseointegration and implant dentistry. His specific interests include understanding the mechanisms behind the interactions among new functionalized implant surfaces, bone substitutes, and biological tissues.

He is a fellow of the ITI, Switzerland, and an active member of the Academy of Osseointegration. He has published more than 50 scientific papers, book chapters and abstracts in international peer-reviewed scientific literature, and he serves as an editorial board member for the *Global Journal of Health Science*.

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Introduction

Osseointegration and implant dentistry research has expanded in so many ways. Basic and clinical research has created a quiet revolution in dentistry and contributed to the development of clinical solutions, simplifying and improving complicated surgical and prosthetic protocols.

In the recent years, methodological developments have enabled a better understanding of the mechanisms of integration of dental implants. Sophisticated techniques of material science include the fabrication of tailored microtopographic and nanotopographic features on real implants, new preparation techniques enabling the visualization and characterization of intact implant–bone interfaces at the atomic level, and new manufacturing principles directed toward the individualization and customization of the next generation of implants.

The modern tools of cell and molecular biology have contributed significantly to the understanding of tissue regeneration. Clinically, new and advanced implants incorporating chemical and topographic information have been introduced. Adjunct and supportive therapies that target biological pathways have emerged, such as platelet, stem cell and progenitor, and growth factor therapies. To meet the demand for predictive preclinical-screening and clinical-monitoring methods, much focus is on the development of noninvasive tools providing structural and functional information about the performance of implants. At the meeting point of the engineering sciences, emerging area of regenerative medicine, and clinical discipline of dental implantology, patients and their needs are the prime focus. The area also attracts considerable interest from the business community, having a strong annual growth.

The *Implant Dentistry Research Guide* is the first comprehensive book providing state-of-the-art scientific and technical information in a clear format and consistent structure, making it suitable for formal course work or self-instruction. It also introduces updated topics and emergent areas of methodology still under active development.

It contains information on both state-of-the-art and emerging methods and techniques used in the studies of relationships between material properties and biology and in the development of tissue-integrated dental implants. It guides the reader through the entire methods, starting from surface characterization analyses, in vitro experiments, ethics and regulations for the use of laboratory animals, to the use of different animal models in dental implant and bone-regeneration research, imaging techniques, computer finite element models, biomechanical methods, analytical methods for the bone–implant

interface, and human trials. The applications of the techniques are illustrated in the form of case studies. The detailed illustrations help avoiding potential ambiguities.

The *Guide* is a much needed resource to improve the knowledge and skills of professionals in the field of implant dentistry. Its contents are as follows:

Chapter I — Osseointegration is the result of bone-formation and bone-regeneration processes, occurring at the bone–implant interface. Although many studies of the mechanisms of osseointegration have been conducted, the underlying cellular and molecular events have not received much attention. The recent developments in research technologies have enabled analyses of the interface between living tissues and implant surfaces at the molecular level. Such technologies can be used at a high degree of precision to understand the osseointegration process including early inflammation, mesenchymal stem-cell recruitment, and cell–cell communication. This chapter illustrates the sequence of molecular interaction, cellular events, and general pattern of osseointegration following implant placement. This information includes the relevant knowledge from the fields of bone biology and osseointegration in an attempt to engage the reader in this interesting area.

Chapter II — The research questions on osseointegration start at the material level: what are the surface properties that enhance osseointegration? Surface topography has long been identified as a crucial parameter for successful osseointegration of oral implants. At present, general guidelines exist on how to measure implant surfaces, and a set of quantitative parameters have, after extensive research, been recommended for topographical evaluation. However, the current implant surfaces are often geometrically complex and include nanoparticles, porosity, coatings, and deterministic patterning, thus increasing the demands for new measurement techniques and evaluation methods. *This chapter provides* the general guidelines for topographical measurement and characterization of implant surfaces.

Chapter III — Of the many surface parameters of dental implants, the surface structure, chemistry, crystal structure, and roughness are the key parameters enhancing the tissue response to implants and, therefore, have often been evaluated. This chapter provides an overview of the approaches and methods to probe the surface structure, chemistry, and oxide thickness of the clinically available titanium dental implants.

Chapter IV — A synthetic material forming a bone-like apatite layer on its surface in the living body can bond to living bone. The *in vivo* apatite formation on these bone-bonding ceramics is reproducible in cellular simulated body fluid with ion concentrations nearly equal to those of human blood plasma. This indicates that the bone-bonding bioactivity of a material can be predicted from the apatite formation on its surface in simulated body fluid. This chapter describes the state of the science and procedures of using simulated body fluid to determine the bioactivity of a new implant candidate material.

Chapter V — The integration of biomaterials *in vivo* depends on the complex interplay of the numerous cell types attracted to the implant surface. This involves not only cell–biomaterial interactions but also cell–cell interactions at the implant surface in which each of these cell types affects the state of differentiation and functionality of the others, either by direct contact or indirectly through released factors. Cell-culture models are routinely used to screen and identify promising new materials and devices before conducting *in vivo* experiments. Cell cultures focus on the morphological aspect, growth capacity, and state of differentiation of cells on materials with various chemical compositions and topographies. *This chapter provides answers* to the commonly asked questions: which cell-culture models

can be used to investigate the process of osseointegration, gain insight into this process at the cellular or molecular level, and discuss what has been learned about osseointegration by using the different cell-culture models.

Chapter VI — New prosthetic components to attach endosseous implants to the supraconstruction are developed by using a wide range of biomaterials and computer-assisted design and/or manufacture. The relationship between the materials and the design of all parts of an implanted medical device are critical and yet remain poorly understood in some areas. Laboratory mechanical testing has been recommended as a preliminary investigative tool for the in vitro evaluation of new materials and medical devices, reducing the number of clinical studies needed to fully characterize the performance of a given implant-restorative system. It is also used to evaluate critical and complex issues in a controlled and repeatable manner. This chapter describes computer simulation by using finite element analysis and in vitro tests utilized to evaluate implant-restorative systems with emphasis on the advantages and potential limitations of each methodology and how the acquired outcomes can be related to the clinical scenario.

Chapter VII — Animals are used in specific areas of research where they do not differ from humans for the characteristics being studied, and where the differences are of no significance to the project. Animals are therefore used as highly specific "models" of humans. The similarities and differences between models must be considered in every project. Several biocompatibility tests for new materials involve animal testing. Animal tests are valuable, but they are often difficult to interpret. This chapter describes a possible method paving the way for an incremental and synergistic approach integrating the existing models based on research conducted in the mouse, rat, rabbit, ovine, swine, dog, and nonhuman primate. The specific aim is to present a tool to be concretely implemented in a self-reflection sheet before starting an animal trial.

Chapter VIII — Small laboratory animals are invaluable models for a variety of human disorders. The rat and rabbit models have been used extensively in biomechanical studies of dental implants. This chapter provides an overview comparison of the strengths and limitations of the commonly available small laboratory animal models frequently used in implant dentistry and related research, explores the advantages and disadvantages of the rat maxilla as a dental implant model, and includes a description of methodologies that may be used to prepare this model in both loaded and unloaded conditions.

Chapter IX — The surgical placement of dental implants is governed primarily by the prosthetic design and secondarily by the morphology and quality of the alveolar bone. Alveolar bone-augmentation procedures are frequently applied in implant dentistry, and can be defined as any attempt to preserve or increase the height or the width of the residual ridge or the repair of defects with grafts or biomaterials. This chapter describes how the use of preclinical animal models represents a necessary step in the development and evaluation of a candidate therapy for alveolar augmentation before application in humans.

Chapter X — Augmentation of the maxillary sinus is a clinically useful technique for severely pneumatized maxillary sinuses before reconstruction with a dental implant. A novel technology for the indications of sinus floor elevation or augmentation has been introduced clinically. Animal models were developed to confirm that the technology meets these essential criteria for dental implant placement, fixation, and long-term retention, mimicking real clinical situations. A variety of animal models have been proposed and used to study

sinus floor-elevation procedures. This chapter provides an overview of the different animal models used in maxillary sinus-augmentation research.

Chapter XI — Animal experiments are subject to strict regulations. This chapter discusses the ethical issues raised by animal experimentation, the solution to the moral dilemma offered by the 3 Rs (*Reduction, Refinement, and Replacement*), a selection of laws controlling animal experimentation, the testing required by regulations of various kinds, and the specific question of dental implant testing.

Chapter XII — Histological analysis by light microscopy is a sensitive parameter and valuable technique for evaluating implants and their surrounding tissues. At the light microscopy level, many studies have been performed on animal experimental implants as well as clinically retrieved implants. Histology can provide unique information on cellularity and dynamic indices of bone remodeling. This chapter describes both the theoretical principles and the practical techniques used in histology to analyze the bone–implant interface.

Chapter XIII — Micro-CT is a nondestructive, fast, and precise technique that allows not only static measurements of three-dimensional bone architecture but also dynamic measurements of failure initiation and propagation as well as damage accumulation. It can provide a spatial representation of bone formation at the implant surface and peri-implant region up to a few microns or even better and enable both qualitative and quantitative morphometry of bone integration around dental implants. This chapter describes the current application of micro-CT in different areas of bone and biomaterials research. Details of studies in the area of implant research are highlighted along with the advantages and disadvantages of micro-CT.

Chapter XIV — The application of electron microscopy in the life sciences has progressed by leaps and bounds over the past decades. Ultrastructural studies of biomaterial–tissue interfaces using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) present unique challenges for technical preparation and scientific interpretation. The recent advances have extended the application of two-dimensional and three-dimensional electron microscopy imaging technologies to reveal complex biological events and species at biomaterial–tissue interfaces. The ability to probe such interactions is tremendously valuable to investigators seeking to design improved biomaterials with enhanced functionality and specificity. Recently, focused ion beam has been introduced in biomaterial and implant research. This technique is generally coupled with a plethora of complementary tools such as dual-beam scanning electron microscopy (SEM), environmental SEM, energy dispersive X-ray spectroscopy (EDX), and cryogenic possibilities. This chapter describes the application of electron and ion beam microscopes for implant surface and bone–implant interface characterizations, with particular emphasis on TEM-based preparation of biomaterials, biological matter, and their interfaces using FIB.

Chapter XV — Molecular biology pertains to the study of living systems at the molecular level, especially DNA and RNA, and provides a background appropriate for further work in the rapidly expanding areas of genomics, cell biology, biotechnology, microbiology, diagnostics, and therapeutics. The analysis of gene expression in particular, through the development of molecular biology technologies, has provided an insight into the fundamental cellular and molecular mechanisms that result in osseointegration. This chapter introduces the molecular biological techniques that can provide the tools necessary to further elucidate the underlying mechanisms of osseointegration, as well as the disease processes that can lead to

implant-related pathology. This understanding may then be translated to clinical practice by providing leads for further implant development based on a sound understanding of the osseointegration process at the molecular level.

Chapter XVI — The application of engineering knowledge to dentistry has helped the understanding of the biomechanical aspects related to osseointegrated implants. The nature of the loading forces and the transfer mechanism of such forces to the biological tissues have been suggested as factors in the development of the implant–tissue interface and, indeed, implant longevity. Several techniques have been used to evaluate the biomechanical load on implants, including photoelastic stress analysis, finite element stress analysis, and strain-gauge analysis. This chapter describes the engineering methods used in dentistry to evaluate the biomechanical behavior of osseointegrated implants.

Chapter XVII — Clinical trials are a set of procedures in medical research that are conducted to enable the collection of safety and efficacy data for health interventions (e.g., drugs, diagnostics, devices, therapeutic protocols). These trials can be conducted only after satisfactory information has been gathered on the quality of the nonclinical safety, and Health Authority or Ethics Committee approval has been granted in the country where the trial is to be conducted. Various types of clinical study designs can be employed to investigate questions about dental implants. Such research can be categorized as nonexperimental descriptive studies or experimental treatment studies. The former includes case reports and cohort, case–control, cross-sectional, and ecological studies, and the latter includes randomized controlled clinical trials and non-randomized clinical trials. These studies are indicated for adopting new innovations and major product changes. This chapter provides an overview of these clinical research designs, along with the strengths and limitations of each approach, and the general principles of randomized controlled trials are discussed in detail, including how these studies can be employed to investigate questions about dental implants.

Chapter XVIII—Inclusion criteria are the parameters to admit a patient in a clinical trial, whereas exclusion criteria are the criteria for excluding patients from a study. The choice of inclusion and exclusion criteria is critical when designing a clinical trial in all the fields of medicine. Ideally, they should be clear and objective, so that any investigator involved in the clinical trial can easily identify the patients who can be enrolled or excluded from the study during the screening visits. This chapter describes the inclusion and exclusion criteria used for defining cases in studies of implants and tissue regeneration.

Chapter XIX — The clinical evaluation of an oral implant is traditionally focused on the healing-in of a material. The rapid introduction of various products and the steeply increasing number of installed implants have resulted in an increase in the number of complications related to oral implants in humans. Therefore, research on complications and destruction of peri-implant tissues demands further development of sophisticated techniques to reveal the causal and modifying factors in these processes. This chapter describes some methods available to monitor the biological performance of a biomaterial in humans.

Chapter XX — Radiography plays an important role in routine clinical practice and research projects evaluating dental implants. It is a commonly used diagnostic tool for monitoring marginal bone loss around the dental implants. This chapter presents some relevant aspects of two-dimensional radiography, with focus on implant research. Specifically, the issues of (a) the reliability of radiographic measurements to estimate the true marginal peri-implant bone level, (b) projection geometry for proper implant image quality and evaluation of the marginal peri-implant bone level, (c) dimensional distortion in

periapical and panoramic images, and (d) the timing of radiographic examination for evaluating the marginal peri-implant bone level during monitoring of implants are discussed.

Chapter XXI — Achievement and maintenance of implant stability are prerequisites for the clinical success of osseointegrated dental implants. Therefore, the measurement of implant stability is an important method for evaluating the success of an implant. Resonance frequency analysis is a technique for noninvasive assessment of implant stability. This chapter presents the current knowledge on resonance frequency analysis and discusses the utility of the technique in dental implant research.

Because the Internet is one of the most powerful tools to share information, an attractive *Web site*—<http://www.implantologyresearchguide.com>—has been created for this book, allowing the reader to provide general feedback on the book, and get updates for new tools and techniques in the field. Finally, an appendix of standard abbreviations is also included.

Ahmed M. Ballo

Part I – Osseointegration Mechanism

Chapter I

Osseointegration: Outlines of Cellular and Molecular Mechanisms

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ABSTRACT

The osseointegration process is typically characterized by the overlap of different stages, including the initial inflammatory response, initial bone formation, osseous anchorage and bone remodeling. Each stage is characterized by a specific set of cellular and molecular events. Advances in molecular biology and biochemistry, cell biology, developmental biology, wound-healing physiology, materials science and engineering, and novel laboratory techniques, have provided incentives and opportunities for renewed and ever-increasing interest in detailed studies of the mechanisms of osseointegration. The aim of this chapter is to illustrate the sequence of molecular interaction, cellular events and the general pattern of the osseointegration process performed following implant placement. Further, the influence of surface modification on cellular and molecular activity at the bone-implant interface is discussed on the basis of recent *in vivo* investigations.

1.1. Introduction

Osseointegration has been and still is a phenomenon subject of a great deal of debate and research. However, the discovery of osseointegration and its clinical application in dentistry by Professor Per-Ingvar Brånemark and his colleagues is considered as one of the most significant and important developments in dentistry. Basically, two responses of bone to implant materials can be distinguished. The first involves the formation of a connective tissue

capsule around the implant, i.e. fibro-osseointegration. [1] Whereas in the second type, bone is in direct contact with the implant surface without any intervening fibrous connective tissue layer, i.e. osseointegration. [2,3].

Several materials (metals, ceramics and polymers) have been used in implant dentistry. [4-8] Before the advent of osseointegration, oral implants were poorly documented and the success rates were quoted without reference to any defined success criteria. [9] Several oral implant designs, such as blades, needle-like screws and so on, have been employed, without adequate clinical reports. Some implant systems, such as hollow implants (ITI), subperiosteal implants and the Tübingen implants, have demonstrated well-controlled and acceptable short-term (5 years) success. However, they have showed less good results after long-term (10 years) follow-up. At the same time, the Brånemark endosseous implant design has demonstrated acceptable 15-year success rates.

Pure titanium and its alloys have been considered the “golden standard” among biomaterials used for bone-anchored implants. The advantageous use of titanium is based mainly on a favorable combination of load-bearing properties and biocompatibility attributed to a thin passive surface oxide layer. [10] When titanium implants are placed in bone, a close structural and functional connection between the bone and the load-bearing titanium implants takes place. [11] However, this direct contact does not necessarily entail actual bonding between the implant surface and bone. Actual bonding of this kind at the interface would be desirable for the stable fixation of the implants over a long period of time. The concept of osseointegration has been further defined at different levels: clinically, [12] anatomically, [13] biomechanically, [14] histologically [11] and ultrastructurally. [15].

Implant surfaces have long been recognized as playing an important role in the process of bone formation during osseointegration. [16-18] Recently, micro- and nano-technology has rapidly advanced surface engineering in implant dentistry. These advances in surface modification technologies have resulted in more complicated surface properties on micro- and nano-meter scales. Despite the large amount of available data relating to the ultimate, positive, bone response to different surface modification, a detailed understanding of the cellular and molecular mechanisms behind this favorable response has only recently emerged. [19,20] Furthermore, it has been clearly stated that the intentional modification of one or more of the surface properties will result in changes in other surface characteristics, including morphology, chemistry, crystal structure, physics and mechanics (Figure 1–1). It is therefore important to identify the specific surface properties that lead to a favorable biologic response.

Primary implant stability is considered to be a critical factor when it comes to obtaining successful osseointegration. Primary (mechanical) stability must be sufficient to enable the implant to resist micromovement until sufficient biologic stability (secondary stability) is established (Figure 1–2). [21] It has been claimed that surface modification accelerates the osseointegration process and improves implant stability in the recipient bone site. [22,23] However, most of the literature that has been published on the biomechanical behavior of modified implants has focused on only a few and primarily the late periods of osseointegration. A proper understanding of interface biomechanical stability and its effects on the long-term success of the implantation procedure may require additional investigations, including the immediate and early stability and extending over the subsequent periods of osseointegration.

Recently, new advances in research technologies have made it possible to analyze the interface between the living tissues and the implant surface at the cellular and molecular

levels. These technologies can be used with a high degree of precision to understand the detailed mechanisms of the osseointegration process, including events of early inflammation, mesenchymal stem cell recruitment and cell-cell communication.

An understanding of the processes of healing, repair and remodeling at the interface is critical for the further development of new surfaces/surface modifications for dental applications. In addition, the new generations of osseointegrated implants may include the addition of active biologic components and/or specific cell types in order to improve the tissue healing response, particularly in compromised clinical situations and in conjunction with high load demands. Ultimately, an understanding of this kind may create new opportunities for osseointegrated applications in other fields such as orthopedics.

This chapter illustrates the sequence of molecular interaction, cellular events and the general pattern of the osseointegration process performed in experimental animal models following implant placement. Relevant knowledge from the fields of bone biology and osseointegration will be integrated with this information in an attempt to engage the reader in this area. One area of particular interest, the influence of surface modification on cellular and molecular activity at the bone-implant interface, is discussed on the basis of recent *in vivo* investigations.

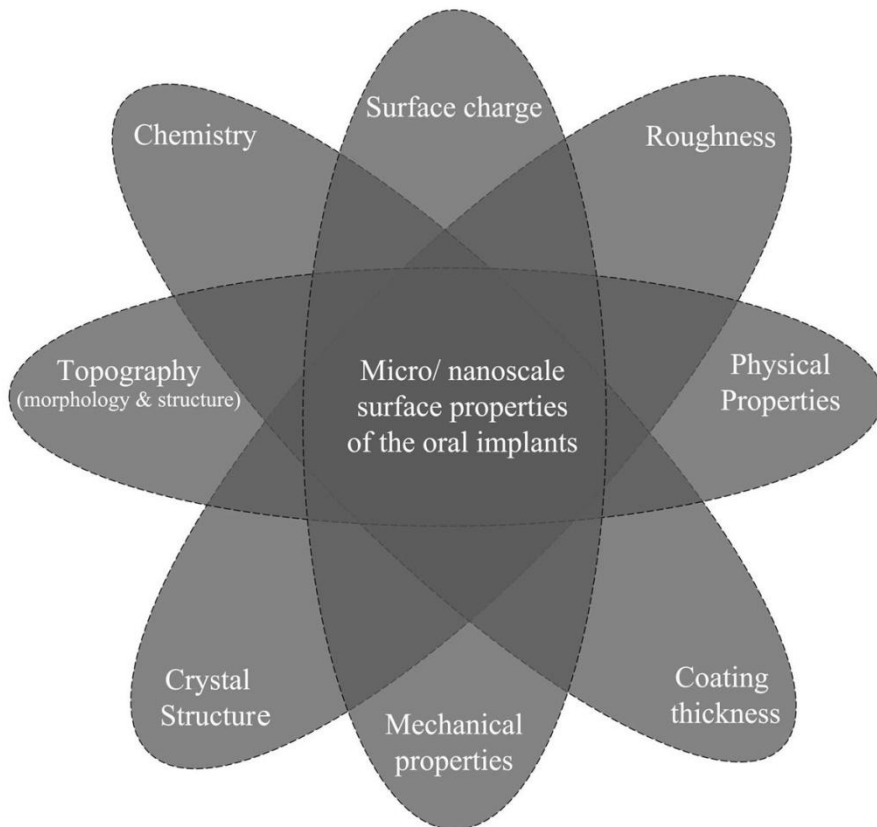


Figure 1.1. Different surface properties of the implant that play important roles in the osseointegration process.

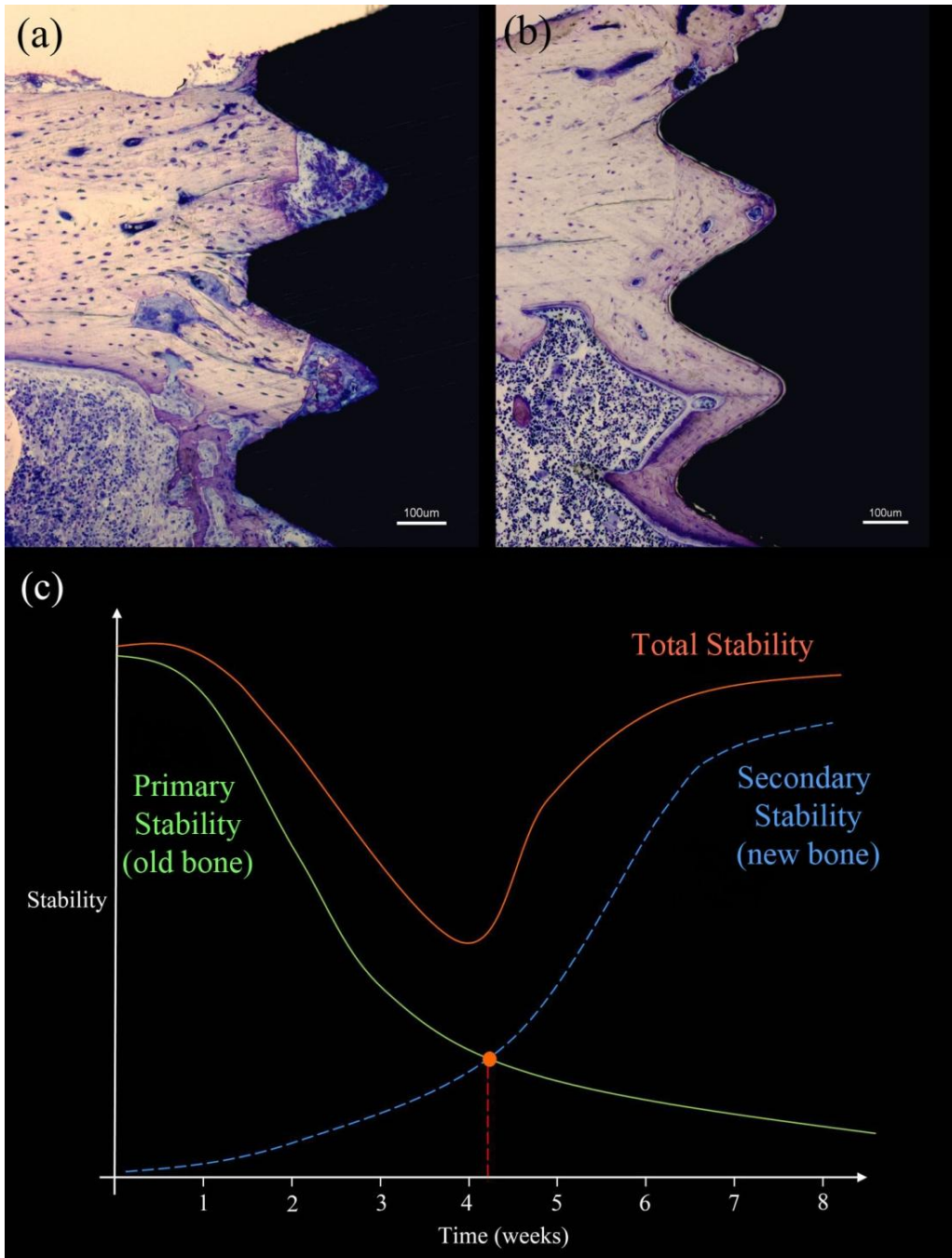


Figure 1.2. (a) High primary (mechanical) implant stability is achieved by the *undersized implant bed preparation* technique. (b) Secondary (biological) stability is obtained by new bone formation. (c) The illustration image shows healing phase duration.

1.2. Osseointegration Processes

Osseointegration represents a unique phenomenon that involves complex interactions between multiple cellular and molecular events that regulate the healing process at the interface. This phenomenon has comparable sequences of events that occur during normal fracture healing through intramembranous bone formation. In addition, peri-implant wound healing shares many prerequisites with normal fracture healing, including precise alignment (anatomic reduction), primary implant stability (stable fixation) and adequate loading during the healing period. [24] The achievement of *primary stability* during the surgical placement of dental implants is one of the success criteria. [25] On the other hand, the bone wound healing events are modulated by the properties of the implanted biomaterial. [26,27]

The osseointegration process is typically characterized by the overlap of different stages, including the initial inflammatory response, initial bone formation, osseous anchorage and bone remodeling. Each stage is characterized by a specific set of cellular and molecular events. It requires the well-orchestrated integration of the complex biological and molecular events of cell migration, cell proliferation and extracellular matrix (ECM) deposition. The cellular responses to inflammatory mediators, such as cytokines and growth factors, and to mechanical forces must be appropriate and precise.

1.2.1. Hemostasis, Cellular Recruitment, Adhesion and Inflammation Stage

Multiple events occur immediately after osteotomy. The peri-implant wound healing process starts with extravasation (bleeding), so that blood is the first tissue that comes in contact with the implant surface after implantation (Figure 1–3). This contact results in a series of immediate and early processes including protein adsorption and coagulation. Erythrocytes, thrombocytes (platelets) and leukocytes are the first cellular participants at the interface and, consequently, a dense fibrin network is formed, resulting in provisional matrix formation (Figure 1–4).

The healing of bone injury requires the continuous influx of various cell types for each ongoing process. The local release of inflammatory mediators, such as the chemicals released from injured tissue (e.g. prostaglandins), products of coagulation and complement (e.g. C5a) and products of fibrinolysis, initiates the cascade that controls early inflammatory events. These events involve the production and release of primary acute phase cytokines (such as TNF- α , IL1 β and IL-6). By activating their target cells, these cytokines generate a second wave of cytokines, including members of the chemokine family. The binding of chemokines to their specific receptors begins a complex biological process, chemotaxis, which involves the rolling, adhesion to and penetration of blood vessels and migration towards the site of highest chemokine concentration. Some chemokines have cellular phenotype specificity, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), which are responsible for monocyte and neutrophil (PMN) trafficking respectively (Figure 1–5). Other chemokines, such as stromal-derived factor-1 (SDF-1) and its receptor, CXCR4, may affect several cell types, including regenerative cells and osteoprogenitors.

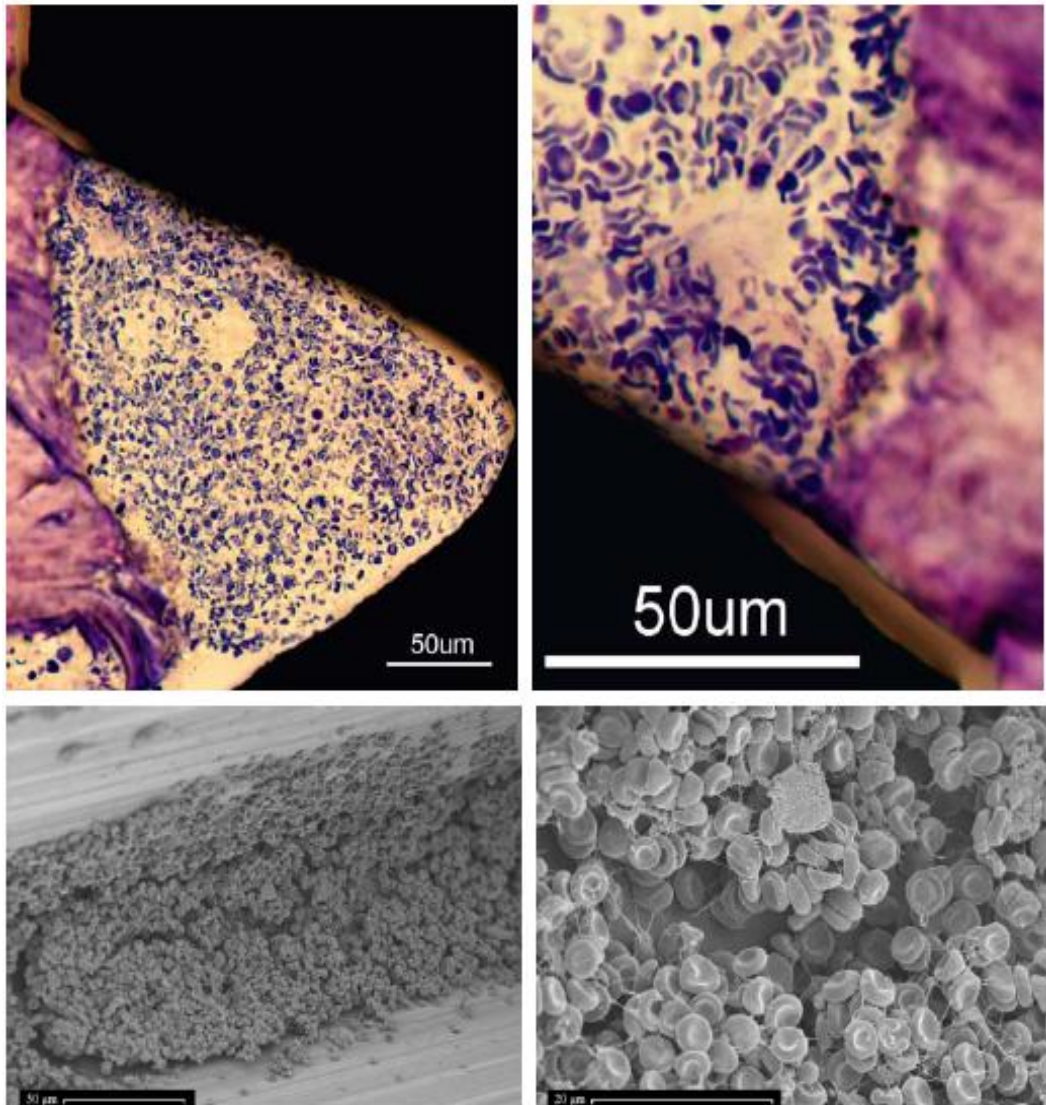


Figure 1.3. Observations after 24 hours of implantation. Upper: undecalcified ground sections of bone interface to machined titanium implants. Bottom: scanning electron microscopy images of machined titanium implants . The area between the implant threads and the surgical bone margin was filled with a fibrin clot including red blood cells.

Subsequent to cell recruitment at the healing interface, cells adhere to the implant surface via specific surface receptors, principally from the integrin family. Integrins are heterodimeric receptors that mediate cell-cell and cell-extracellular matrix (ECM) attachment. They also play important roles in controlling cellular shape and mobility and regulate the cell cycle. Integrins are thought to be expressed by virtually every cell type. Cells of the osteoblastic lineage predominantly express $\beta 1$, $\alpha 4$, $\alpha 5$ and αv integrins in various combinations, while the osteoclast exhibits higher levels of $\alpha v \beta 3$ complexes. [28] On the other hand, at least 13 integrins are expressed by leukocytes, among which $\beta 2$ is a unique leukocyte-specific integrin, [29] with putative roles in leukocyte chemotaxis, phagocytosis and other adhesion-

dependent processes. [30] The $\beta 2$ integrin has also been shown to be expressed by monocytes committed to the osteoclast lineage. [31].

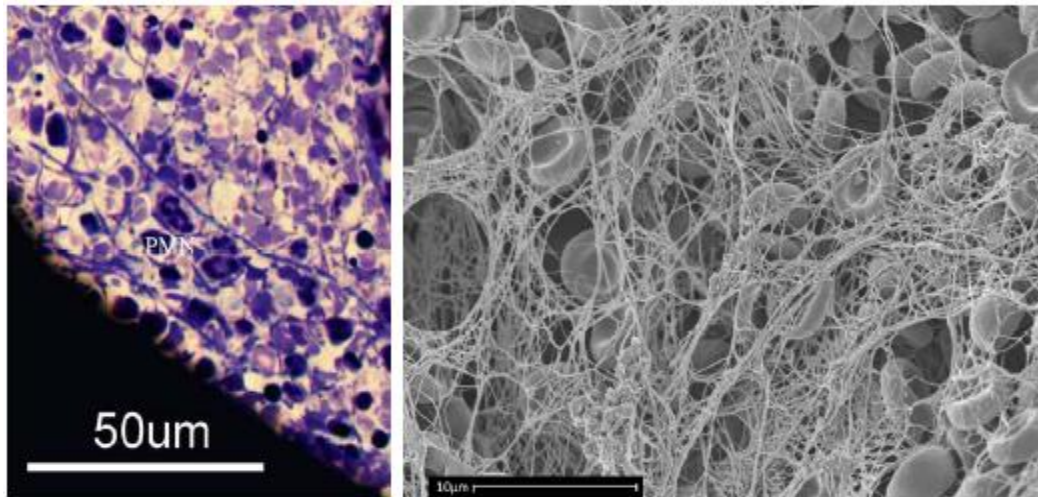


Figure 1.4. Observations after 3 days of implantation. Left: undecalcified ground sections of bone interface to machined titanium implants. Right: scanning electron microscopy images of machined titanium implants. The area between the implant threads and the surgical bone margin was infiltrated by erythrocytes and entrapped leukocytes, mainly neutrophils.

The initial inflammatory stage appears to begin immediately and may last for up to 3-5 days after implantation. Macrophages and multinucleated cells with a monocytic lineage were detected at the bone interface with machined titanium and HA implants during the early phases after implantation. [32-34] These inflammatory cells synthesize cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), which may be important for the regulation of the early events in the osseointegration process. TNF- α and IL-1 β are major proinflammatory cytokines secreted primarily by hematopoietic immune cells, such as monocytes/macrophages and neutrophils, and also by cells with a mesenchymal osteogenic lineage. [35] They have a wide range of effects and can either trigger cell death or promote cell survival, depending on the specific cell surface receptor they bind, the cell type and the intracellular signaling cascade that is subsequently activated. [36]

In relation to bone injury, studies suggest that osteoblasts are removed from the injury site via coordinated apoptosis during bone healing [37] and evidence suggests that IL-1 β may have a role in mediating the appearance and disappearance of osteoblasts. [38] Moreover, positive effects of proinflammatory cytokines on osteoclastic differentiation have been documented. The critical roles of TNF members (RANKL), the members of the TNF receptor family (OPG) and the direct effect of TNF- α and IL-1 β on osteoclastogenic differentiation have been largely investigated. [39] In general, proinflammatory cytokines have been considered to be crucial regulatory components during bone healing. The expression of these cytokines significantly increases during the initial inflammatory phase after bone injury and they show peak expression within the first 24 h following fracture. [40] Their levels are seen to reduce during the regenerative phase and increase again during the remodeling phase. [40]

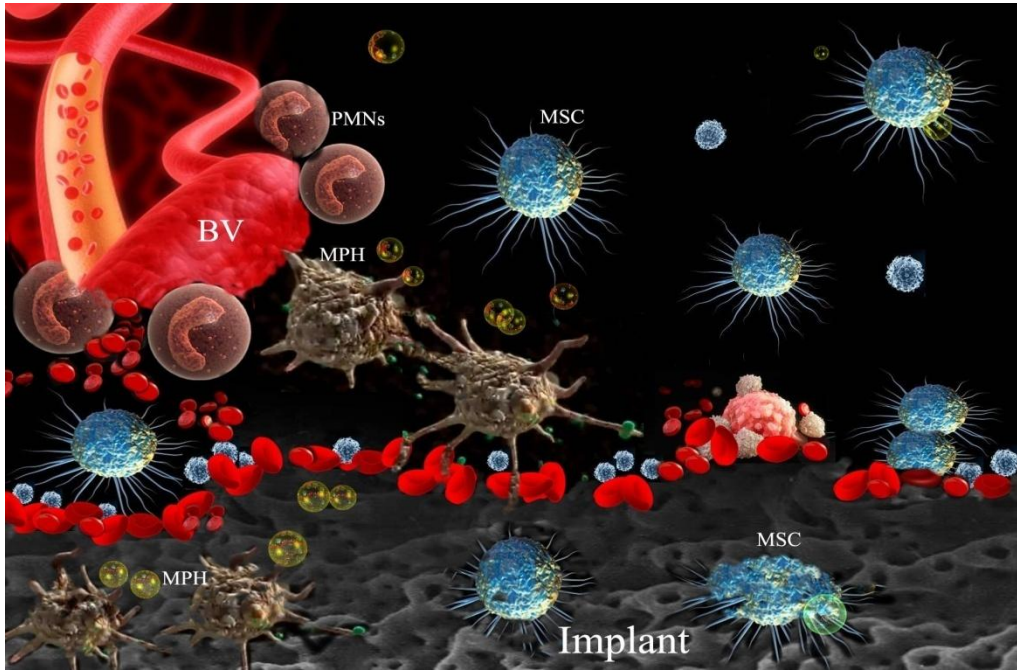


Figure 1.5. A schematic diagram illustrates the bone-implant interface, showing a possible line of interaction between cells and implant surface during the initial phase of healing (BV indicates blood vessel), (MSC indicates mesenchymal stem cell), (PMNs indicates Polymorphonuclear leukocytes) and (MPH indicate macrophages).

1.2.2. Bone Formation Stage

Under the control of specific growth and transcription factors, mesenchymal stem cells differentiate towards the osteogenic lineage during a number of developmental stages, starting from commitment to osteoprogenitors, through preosteoblasts and osteoblasts and finally osteocytes or lining cells. [41] The exact origin of the osteoprogenitors involved in the osseointegration process has not been fully characterized. It is possible that they migrate from the osteogenic endosteal layer of the osteotomy site, from special niches within the bone marrow compartment and/or from the extravasated blood. A wide range of cytokines and growth factors control the differentiation of osteoprogenitors to preosteoblasts and osteoblasts. They include, but are not limited to, transforming growth factor-beta ($TGF-\beta$), bone morphogenetic protein-2 (BMP-2), insulin-like growth factor-I (IGF-I), fibroblast growth factor (FGF), parathyroid hormone-related protein (PTHrP), vitamin D (1,25(OH) $_2$ D $_3$), leptin and members of the interleukin-6 (IL-6) family. [42] The regulation is precisely controlled by specific transcription factors that ensure osteogenic differentiation and subsequent bone matrix formation and mineralization. Runt-related transcription factor 2 (cbfa1/Runx2) is regarded as a master gene for osteogenic differentiation and plays a major role in maintaining the osteo-phenotype. [43] Furthermore, other signaling pathways, such as the mitogen-activated protein kinase (MAPK) system and the Wnt and Smad signaling pathways, are involved. [44].

The transcriptional control of Runx2 is required for the commitment of osteoprogenitor cells to the osteoblast lineage and to exclude options for divergence towards other lineages. [45] It has been clearly demonstrated that Runx2 is essential for *in vivo* bone formation. [46] The binding elements of Runx2 are present in the promoter region of collagen I, OPN, BSP and OC genes. The activation of Runx2 by MAPK via the stimulation of integrin $\alpha 2 \beta 1$, [47] for example, results in the translocation of Runx2 into the nucleus, where it triggers the expression of the osteoblastic genes during the early stage of osteoblast differentiation.

Osteoprogenitors colonize on the pre-existing bone and possibly on the implant surface (Figure 1–6) and continue to differentiate towards the osteoblastic lineage. The preosteoblasts represent a transitional stage between the highly proliferative osteoprogenitor cells and the mature osteoblast. [41] Despite their low production of matrix proteins, preosteoblasts still have the ability to divide. Studies have also shown that preosteoblasts can produce collagen I precursors [48] and express a panel of early bone formation markers such as alkaline phosphatase (ALP), growth factor receptors, several integrins and osteoblast specific factor-2 (periostin). [48,49] Preosteoblasts differentiate into osteoblasts, which are typically cuboid in shape and actively and ultimately secrete the organic bone matrix. The early, not yet mineralized, secretion of osteoblasts, osteoid, contains collagen type I and other non-collagenous proteins, including osteopontin (OPN), bone sialoprotein (BSP) and osteocalcin (OC).

During the early stage of bone formation, osteoblasts express high ALP activity. They also express growth factors and molecules involved in auto- and paracrine regulation and cell-cell interaction. The osteoid seams are located within the cell-rich region between the implant surface and the surgical margins of bone. It also forms directly on the implant surface (Figures 1–7, 1–8) within the bone marrow compartment. Collagen type I provides a scaffold for the growth of bone, facilitates the deposition of other matrix proteins and acts as a nucleation site for hydroxyapatite crystals.

The pattern of the woven bone formation depends largely on the distribution of the capillaries, forming a network of thin trabeculae of woven bone. The majority of trabeculae surfaces are covered by a layer of osteoid and rows of osteoblasts (Figure 1–7).

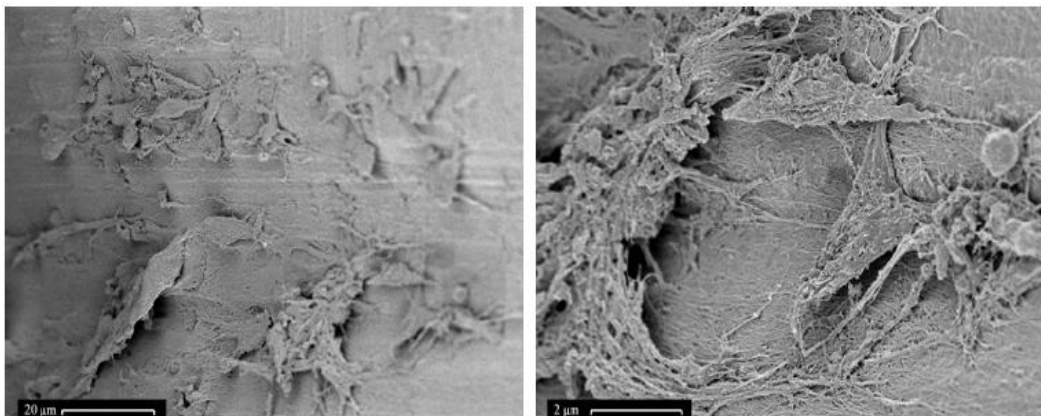


Figure 1.6. Observations after 3 days of implantation. Scanning electron micrographs of retrieved machined titanium implants. Images show mesenchymal-like cells adherent to the implant surfaces. The cells assume flattened morphology, with well-defined cytoplasmic processes oriented along the surface grooves.

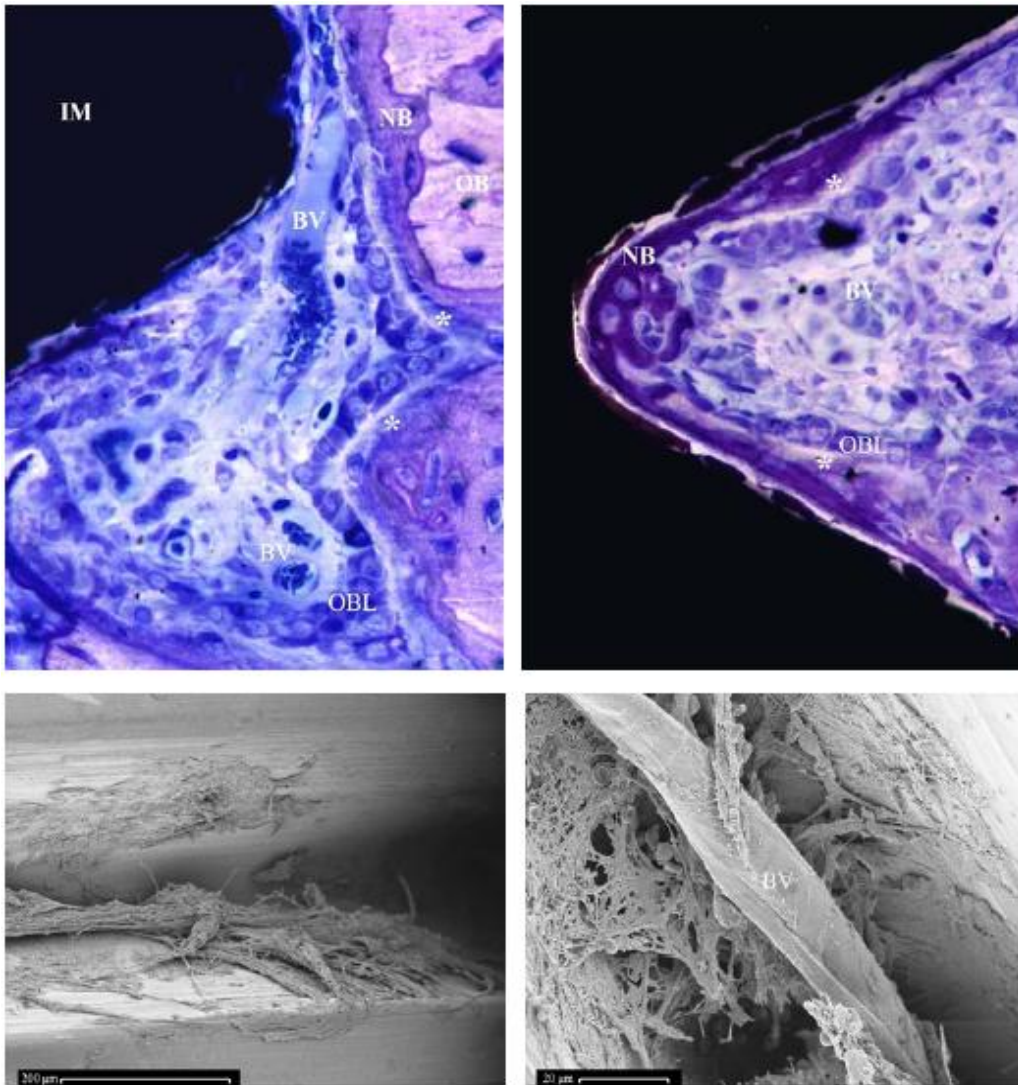


Figure 1.7. Observations after 6 days of implantation. Upper: undecalcified ground sections of bone interface to machined titanium implants. Bottom, scanning electron microscopy images of machined titanium implants. First signs of woven bone and capillaries begin to form. (OB indicates old bone), (NB indicates new bone), (BV indicates blood vessels), (*) indicates osteoid), (OLC indicates osteoblast-like cell) and (IM indicates implant).

The organic matrix continues to mineralize and the osteoblasts move backwards, away from the advancing mineralization front. However, they are sometimes unable to escape and become embedded as osteocytes within bone lacunae. [50] The newly formed bone continues and extends from the endosteum onto the implant surface (distant osteogenesis) and also projects along the surface of the implants (contact osteogenesis) (Figure 1–8) and, as a result, the implant itself acts as an osteoconductive substrate, reducing the size of the defect to be bridged by newly formed woven bone.

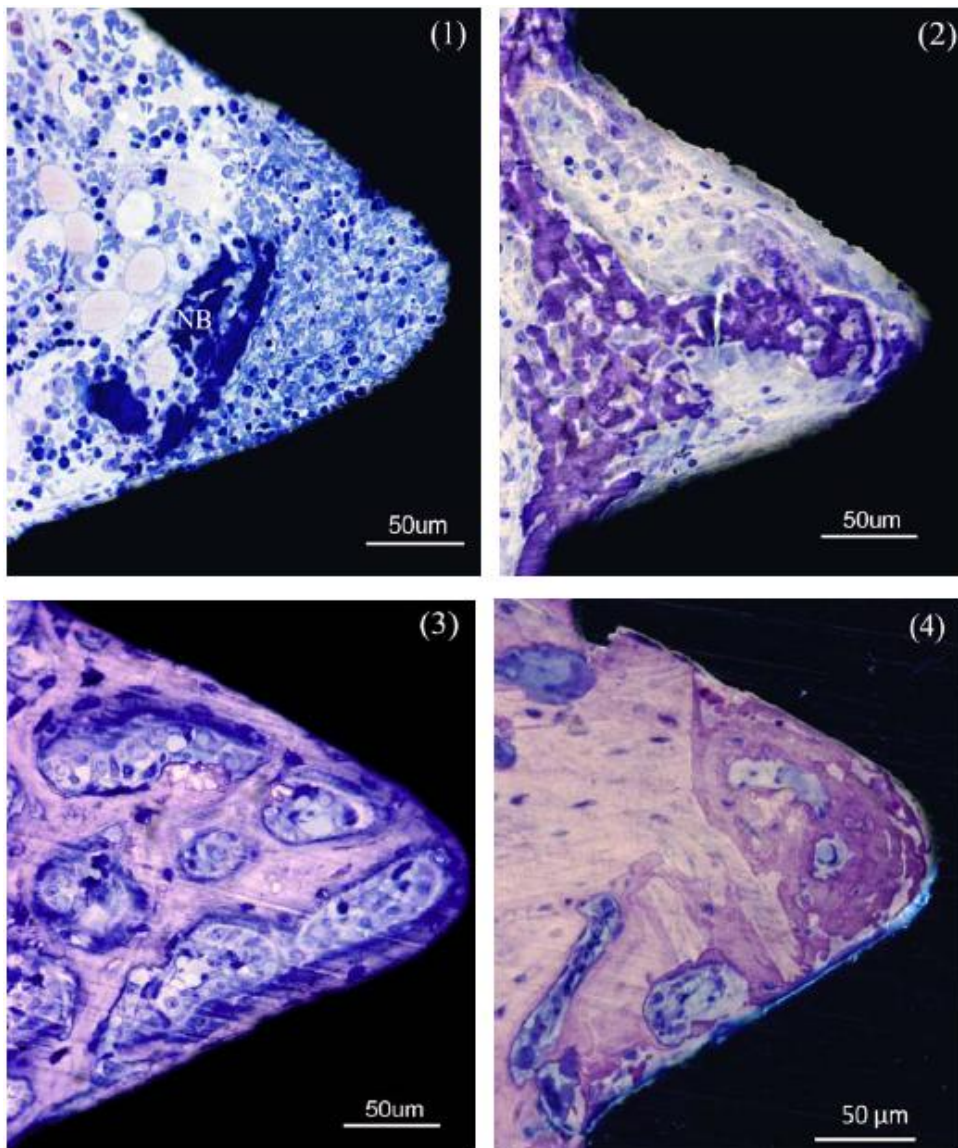


Figure 1.8. Undecalcified ground sections of bone interface to machined titanium implant. Woven bone reaches the implant surface and continues to form along the surface of the implant, using the implant as a template. (NB indicates new bone).

As the healing continues, the implants establish structural osseointegration, as a large part of the implant surface is in tight contact with mature lamellar bone. A few areas at the implant still interface with marrow spaces with cells and blood vessels delimited by the new bone on one side and the titanium surface on the other.

During the course of bone formation, metabolically active bone-forming cells require a blood supply and angiogenesis is therefore essential. The importance of angiogenesis in the process of peri-implant bone formation is increasing. [51,52] Angiogenic factors are expressed concomitantly with matrix metalloproteinases. Metalloproteinases degrade the extracellular matrix surrounding the pre-existing capillaries, allowing for the sprouting of new

vessels (Figure 1–7). [53] The degradation of the extracellular matrix results in the release of angiogenic factors stored within the matrix. Pre-existing osteoblasts are a major source of vascular endothelial growth factor (VEGF). [54].

VEGF is an important mitogen for endothelial cells [55] and it stimulates endothelial cells within the pre-existing capillaries. These cells undergo cell division and migrate to form new vessels. PDGF, angiopoietin and the basic fibroblast growth factor (bFGF) have also been shown to be important factors for angiogenesis. [53] The essential step of angiogenesis preceding bone regeneration was evidenced in the peri-implant setting in healthy bone. [56] Compromised bone condition has shown the delayed upregulation of the angiogenic markers VEGF and endothelial PAS domain protein 1 (Epd1), accompanied by the delayed expression of bone formation markers, collagen type I alpha 1 (COL1A1), bone osteocalcin (Bglap) and bone morphogenetic protein 2 (BMP-2). [56].

1.2.3. Bone Remodeling Stage

The remodeling of the bone *around a dental implant* continues throughout the lifetime of the implant. *It follows similar modeling and remodeling mechanisms of bone adaptation* during skeletal growth, development and response to *physiologic* stimuli and it is composed of two consecutive and interrelated physiologic activities, namely bone resorption and bone formation.

The remodeling and replacement of the woven bone by lamellar bone takes place at a late stage, as does overlapping, to the stage of bone formation during bone healing around the dental implant (Figure 1–8, 1–9). The woven bone forms rapidly and consists of loosely packed collagen fibers of varying size in a random spatial alignment. In contrast, lamellar bone formation takes place more slowly and the collagen fibers are organized in thicker bundles and oriented in the plane of the lamella with regularly arranged osteocytes. This structure makes the lamellar bone mechanically stronger than the woven bone. [57] Ultimately, a layer of mature lamellar bone close to the implant surface becomes thicker than that observed in the early stage. Bone marrow fills the spaces between the bone trabeculae. The osteocytic lacunae at this stage appear smaller than those observed in the early bone formation stage and some osteocytes can be observed at the bone-implant interface (Figure 1–10).

Osteoclasts, which are responsible for resorption, are derived from a hematopoietic origin and are primarily involved in bone resorption. Key cytokines are crucial for the process of osteoclastogenesis and osteoclast development. Several investigations have shown that osteoclastogenesis is critically dependent on macrophage-colony stimulating factor (M-CSF) and the receptor activator of nuclear factor-kappaB ligand (RANKL). [58-60] M-CSF is thought to be critical for the proliferation of the osteoclast precursors, whereas RANKL appears directly to control the differentiation process upon binding to the receptor activator of nuclear factor- κ B (RANK) expressed on the surface of osteoclast precursors. [57] The binding of M-CSF and RANKL to M-CSF receptors and RANK respectively initiates an intracellular cascade and stimulates a series of events inside the cell, leading eventually to the development of mature osteoclasts.

In addition to M-CSF and RANKL, pro-inflammatory cytokines, tumor necrosis factor alpha (TNF- α) and a member of the TNF receptor family, osteoprotegerin (OPG), have been

shown to encompass crucial positive and negative roles respectively in osteoclast differentiation. [58] It is worth noting that marrow stromal cells and their derivative osteoblasts express most of these cytokines and growth factors which are absolutely essential for osteoclastogenesis. Furthermore, some of these ligands, particularly RANKL, are membrane bound, which indicates that the differentiation of osteoclasts requires the direct interaction of bone cells, osteoblasts, with the osteoclast precursors. These interactions outline the mechanisms by which the processes of bone resorption and formation are finely coupled between osteoclasts and osteoblasts during the remodeling process.

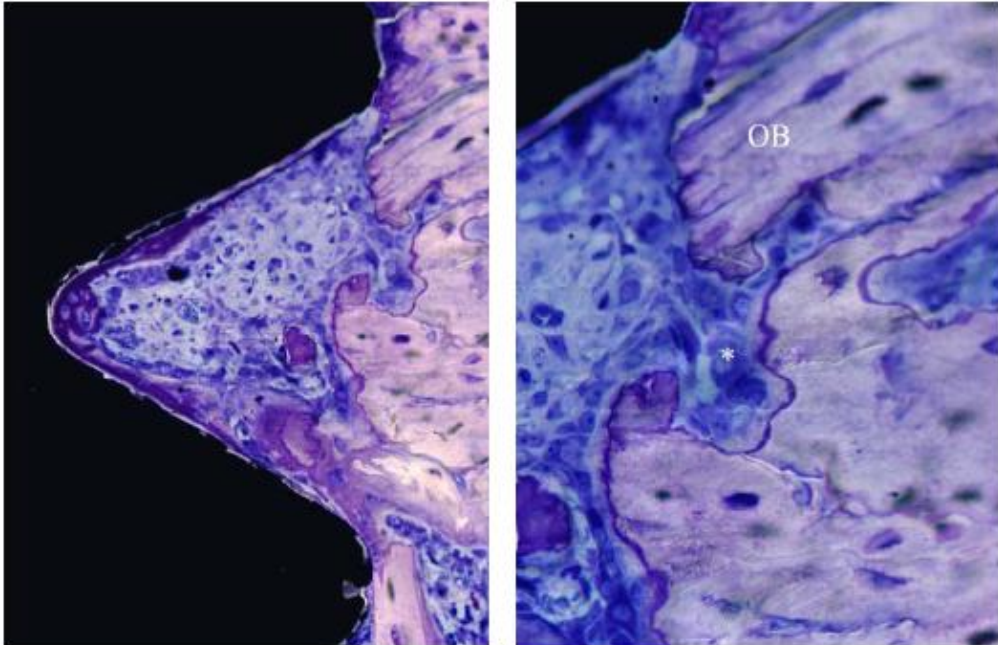


Figure 1.9. Observations after 3 days of implantation. Undecalcified ground sections of bone interface to machined titanium implant. Osteoclast activity at the surgical margin of bone. (* indicates osteoclast), and (OB indicates old bone).

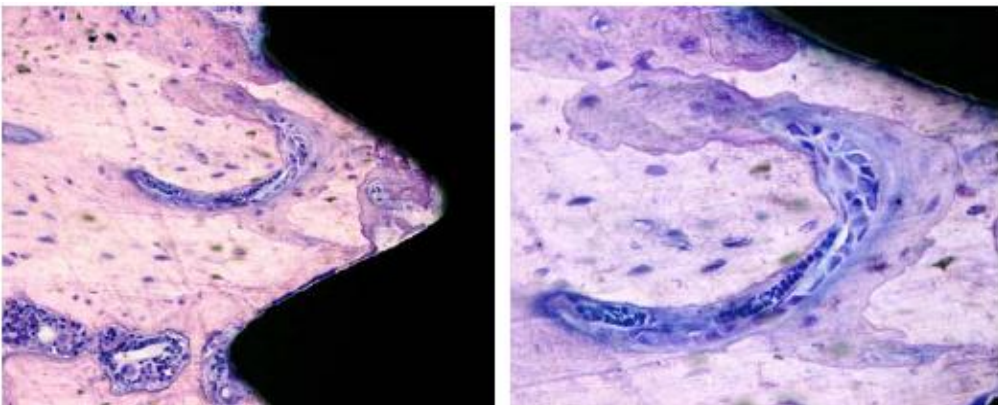


Figure 1.10. Observations after 28 days of implantation. Undecalcified ground sections of bone interface to machined titanium implant. Osteoblast activity was observed near the surface of the unloaded implant, with evidence of some bone deposition over the resorbed surface.

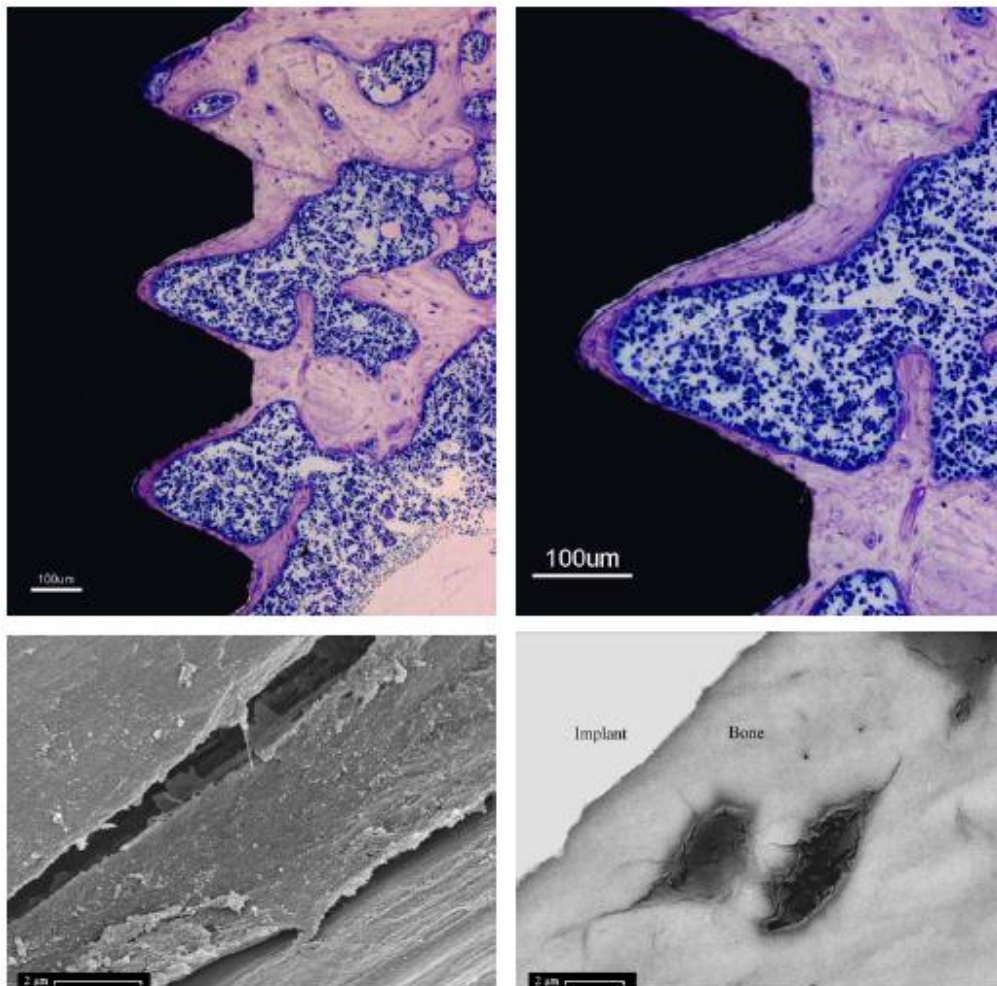


Figure 1.11. Observations after 28 days of implantation. Upper: Undecalcified ground sections of bone interface to machined titanium implants. Bottom (left): scanning electron microscopy image of machined titanium implants. Bottom (right): Backscattered electron microscopy images of bone interface to machined titanium implant. Implants were seen to be well osseointegrated after 28 days of healing, as most of the implant surface was in tight contact with mature lamellar bone and regular osteocytes were recognizable at the bone-implant interface.

During bone resorption, osteoclast precursors are recruited and subsequently differentiated at the site of prospective bone resorption. It is not clear how specific sites are chosen for resorption and how the earliest steps in resorption begin. On maturation, osteoclasts predominantly express $\alpha\text{v}\beta 3$ integrins, which recognize exposed, specific sequences of some bone proteins such as OPN and BSP. [61].

The binding of integrins forms a tightly sealed zone under which bone resorption proceeds by creating a highly acidic microcompartment in which the dissolution of the mineral phase takes place, followed by the enzymatic degradation of organic components by lysosomal proteases such as cathepsin K (CATK). Bone resorption is followed by bone formation, as the osteoclasts leave the resorption pits. The signals that lead to the recruitment and differentiation of osteogenic cells, for subsequent bone formation, are unclear. It is

possible that the release of growth factors that are crucial for bone formation, such as TGF- β 1, IGF-I and BMP-2, from the dissolved matrix plays an important role in providing directional information for osteoblastic bone formation. Furthermore, there are direct effects by osteoclasts on osteoblastic cells, by secreting growth factors, such as TGF- β 1 [62] and IGF-I, [63] or by cell-cell contact. [64].

The importance of osteoclasts and bone remodeling at implants has either been neglected or regarded as processes in the late stage of bone healing at titanium implants. However, *in vivo* morphological studies have revealed that bone remodeling is an integral early component after implantation, irrespective of implant surface properties. [65].

1.3. Effect of Titanium Surfaces on Cellular and Molecular Activity during Osseointegration

Bone regeneration around titanium implants has classically been regarded as being similar to that observed after injury or fracture. This healing is based traditionally on the succeeding phases of homeostasis, inflammation, regeneration and remodeling, with a possible overlap on certain occasions. The distinct phases of inflammation, bone regeneration and bone remodeling following injury or during osseointegration have been well characterized histologically. However, the detailed cellular and molecular activity underlying these phases and the effect of the titanium surface on these events is still lacking. Furthermore, an enhanced understanding of temporal gene expression profiling and the identification of significant biologic molecules related to the distinct phases of healing will greatly advance our knowledge on osseointegration mechanisms and may improve future advances in osseointegrated implants. [66].

In contrast to fracture healing, the cellular and molecular activity that determines the biologic responses at different implant surfaces is largely unknown. Even though bone healing during osseointegration is thought to simulate fracture healing, the presence of biocompatible but not inert titanium implants with distinct surface properties may influence the different processes. [67] Different surface alteration techniques, such as anodization, blasting, etching and surface coating, and combinations of some of these techniques are being used increasingly and are claimed to induce prompt tissue healing and stronger bone formation compared with the original machined implant surface. While these assertions have largely been addressed morphologically and biomechanically, the potential differences in *in vivo* cellular and molecular responses to these surfaces have not been established.

Our existing knowledge of the healing process at different titanium implants has been largely acquired from histological and biomechanical data and correlations with normal fracture healing. The histological and biomechanical studies present strong evidence that bone will respond differently if the implant surface properties are modified. [66] Consequently, a great deal of attention has been paid in *in vitro* studies of the cellular and molecular activity on different substrates to extrapolating the results to the actual interface between implant and living bone tissue. The results of these *in vitro* studies indicate that the surface influences the initial sequences of protein adsorption, platelet adhesion and hemostasis, complement activation, inflammation and osteogenic cell response. [68-71] Bearing in mind the important information acquired from these studies, however, they remain to a large extent

unrepresentative of the actual scenario of *in vivo* implantation. For example, *in vivo* investigations describe the recruitment of different cell types and at different stages of morphological differentiation prior to bone formation at the titanium implant surface. [72,73] However, the role of various cell types, whether from an osteogenic or other lineage, in osseointegration has not been clearly described. Many questions need to be addressed; they include how early molecular and cellular events are stimulated by material surface properties *in vivo*; how such events influence the organization of the surrounding tissue and its interlocking or bonding with the implant surface; and, in turn, the stability of the implant within the surrounding bone tissue for continuous load interactions.

The developments in research technologies are providing new opportunities for introducing molecular techniques to investigate the interface zone between bone tissue and implant surface. Large-scale cellular and molecular studies of the interfacial processes during osseointegration reveal that the gene expression of the implant-adherent cells is instantly and differently influenced by titanium implant surface properties. [74,75] The *in vivo* gene expression analyses were combined with immunohistochemistry and scanning electron microscopy (SEM) to provide spatial information about the different cell phenotypes at the interface. These studies eventually showed that the inflammation, bone formation and bone resorption processes strongly influence the biomechanical strength of the bone-implant interface. [76]

The processes of initial cellular recruitment and adhesion to titanium surfaces have not received the same kind of attention as that given to the mere bone formation process. In spite of this, inflammatory cells such as monocytes/macrophages can be seen at a very early stage at the bone interface with titanium and HA surfaces [34] and they have been regarded as a pivotal cell lineage both during successful osseointegration and in the pathogenesis of implant failure. [48] The activity and release of mediators from macrophages adhering to the implant surface may influence the regeneration process at that surface. In soft tissue healing around titanium implants, the data present strong evidence of the modulation of inflammatory cell responses by titanium surface roughness and composition. [77,78] The local release of inflammatory mediators, in addition to the modulatory role of the implant surface, initiates the cascade that controls early inflammatory events.

During the first 24 hours after implantation in bone, a higher level of expression of MCP-1 was revealed at machined implants compared with anodically oxidized implants. [74] The machined implant was also associated with the increased expression of pro-inflammatory cytokines TNF- α and IL-1 β during the period from 3 hours to 6 days after implantation. [74,75] The similarity in the expression profiles of MCP-1 and the pro-inflammatory cytokines indicated that the recruitment of inflammatory cells was accompanied by cytokine activity at both machined and oxidized surfaces, with greater magnitude at the machined ones. The immunohistochemical observations for the same time period showed CD163-labeled monocytes/macrophages at both implant surfaces. However, large amount of fibrinous material covered the machined implants and was observed up to 6 days of implantation (Figure 1–12). Fibrin has been described as augmenting the pro-inflammatory response to biomaterials, [79] which might explain the upregulated pro-inflammatory cytokine expression at the machined surface as early as 3 hours and continuously during the early phase of osseointegration. [74,75] Interestingly, the acid treatment of the titanium surfaces appears to influence the inflammatory response at a somewhat late stage, where hydrofluoric acid-

treated titanium implants resulted in the significant expression of anti-inflammatory cytokine IL-10, between 4 and 8 weeks, compared with untreated implants. [80,81]

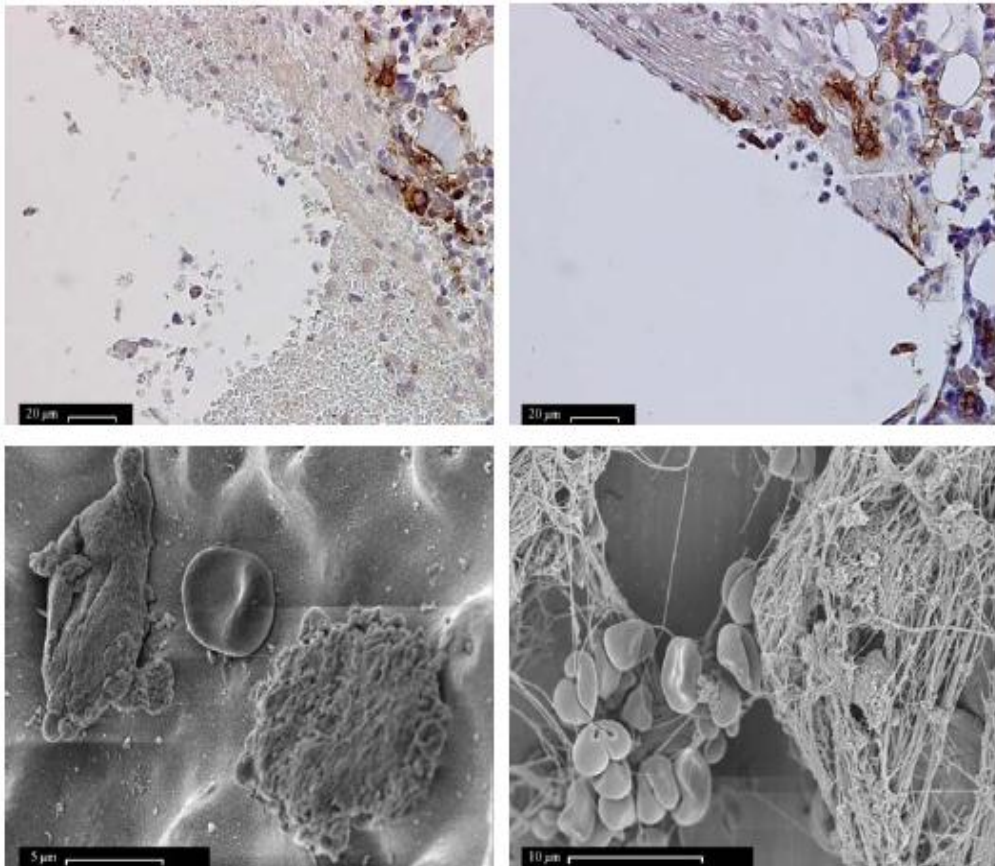


Figure 1.12. Observations after 24 hours of implantation Upper: immunohistochemical sections of the interface showing CD163-positive monocytes/macrophages in the interface zones of oxidized (left) and machined (right) titanium implants. Bottom: scanning electron micrographs of oxidized (left) and machined (right) implants. Biologic material adhering to the machined implant was highly fibrinous, with numerous erythrocytes and leukocytes compared with the oxidized implants.

Chemokines attract different cells depending upon the chemokines and/or chemokine receptors that are expressed [82] and, furthermore, they are implicated in tissue repair. [41,47] The chemokine receptor CXCR4 plays a critical role in the homing and mobilization of different stem cell lineages [42,43,83] including MSCs. [48,84-86] One of the main findings during the initial 24 hours after titanium implantation in bone was the significant modulation of the chemokine receptor CXCR4 by the implant surface properties. [74] These results provided evidence of the role of this chemokine receptor during the early events of osseointegration. After 12 hours of implantation, CXCR4 expression was shown to be about 11 times higher at the oxidized implants compared with the level at machined implants. Together with its exclusive ligand, stromal derived factor-1 α (SDF-1 α), CXCR4 has recently attracted significant attention as a major axis for the local and systemic recruitment of MSCs to sites of tissue repair and regeneration. [87-90].

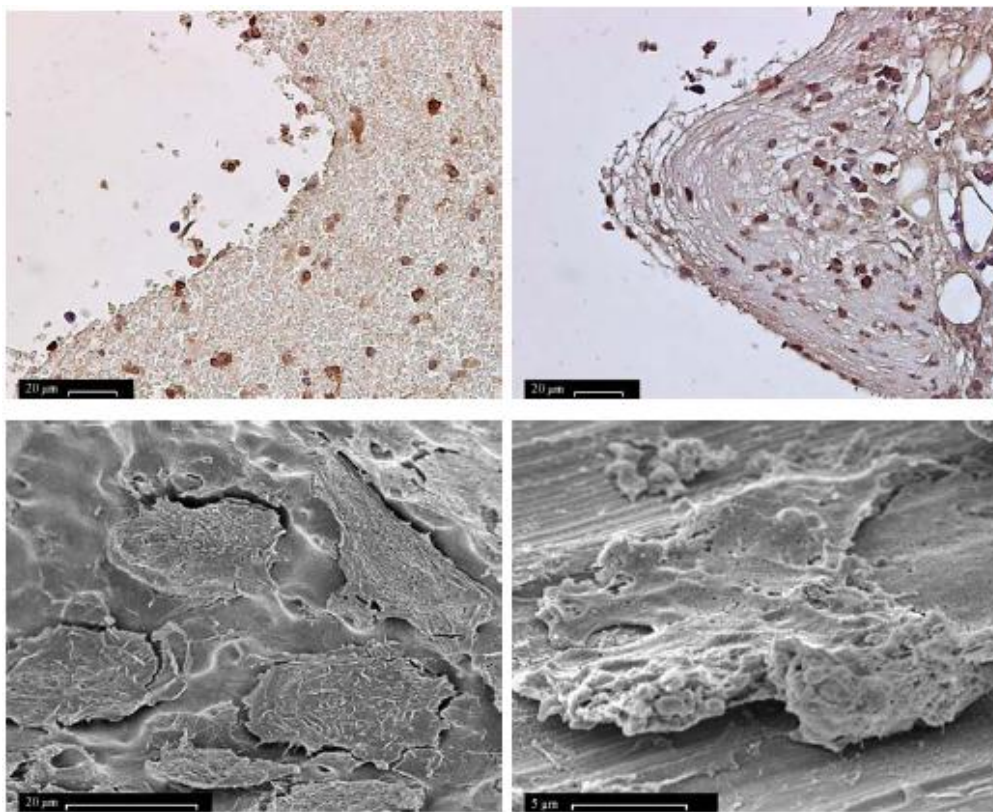


Figure 1.13. Observations after 24 hours of implantation. Upper: immunohistochemical sections of the interface showing periostin-positive mesenchymal and osteoprogenitor cells at oxidized (left) and machined (right) titanium implants. Bottom: scanning electron microscopy of oxidized (left) and machined (right) titanium implants. On the oxidized implants, mesenchymal cells were frequently seen.

The blocking of CXCR4 significantly inhibited the *in vivo* migration of circulating osteoblast progenitor cells to subcutaneously implanted collagen pellets. [91] Furthermore, recent findings demonstrate that MSC migration to a fractured tibia site is highly dependent on CXCR4. [92] The likely role of the chemokine CXCR4 at the oxidized implant surface was corroborated by immunohistochemical and SEM observations showing the predominance of mesenchymal stem cells at that surface (Figure 1–13).

Another principal event during the initial and early period of implantation is cellular attachment to the implant surface. At the *in vivo* bone-implant interface, this attachment is mediated via a protein-rich layer to which cells adhere using specific combinations of integrin receptors. Several *in vitro* studies have demonstrated a major influence by material surface properties on the patterns of cell attachment and integrin expression. [93-99] To date, few *in vivo* data have been provided on the role of integrins in cell attachment at implant surfaces. The surface-dependent regulation of the expression of integrins may reflect the temporal changes in the interfacial matrix/tissue organization phases which are principally influenced by the physico-chemical properties of the surface. Cells adhering to oxidized surfaces revealed the upregulation of integrin- β 1, - β 2 and α v during the first 24 hours of implantation. [74] From these studies, integrin- β 1 appears to be a critical receptor, not only for cellular

contact and communication at the interface zone but also for osteogenic differentiation and the process of bone formation. [100- 103]

The increased expression of integrin- β 1 exhibited an association with CXCR4 at the oxidized implants, which, together with the higher number of mesenchymal cells, as shown in the SEM observations, and the increased expression of osteogenic markers, such as ALP and OC, suggested that the surface-modified implant was associated with the higher recruitment of MSCs through mechanisms which involve the modulation of CXCR4 chemokine receptors and integrin- β 1 expression (Figure 1–13).

The surface properties of the titanium implant have also influenced the expression of integrin- β 2, which is known to be expressed mainly by leukocytes [104] and not by cells with an osteoblastic lineage. [105] Integrin- β 2 was higher at the oxidized implants after 12 hours of implantation. This integrin has also been shown to be expressed by osteoclast precursors. [106] Findings showing a higher expression of osteoclastic markers (cathepsin K; CATK) at the oxidized surface [75] suggested that the higher expression of β 2 integrin may be due to the early recruitment of osteoclast progenitors and preosteoclasts at the interface.

Focusing on the osteogenesis-related genes, the peak expression of ALP, OC, on machined and oxidized implants, was observed 3 days after implantation. [75] The activity of early bone formation at both implant types was determined immunohistochemically by confirming a high signal for periostin, a marker of intramembranous bone formation, at the 3-day period of implantation. Interestingly, a concomitant peak for remodeling-related genes, tartrate-resistant acid phosphatase (TRAP) and CATK, was also detected on day 3 after implantation in rat bone. [75] Previous studies of the bone fracture model (without implants), in similar animal models, have shown that ALP and OC expression attained their peaks after 5 and 11 days respectively. [107] Osteoclastic gene expression levels of TRAP and CATK have been shown to attain their earliest peaks between 7 and 14 days. [108,109] The observations that the peak expression of osteogenic and osteoclastic markers was detected as early as 3 days at the implant surface and that oxidized implants were associated with significantly higher levels than machined implants indicate, firstly, that bone remodeling around implants starts much earlier than has previously been assumed and, secondly, that the implant surface has an influence on the level of expression of osteogenic differentiation and bone formation and remodeling genes.

During the early bone-formation phase, MSCs migrate, proliferate and undergo differentiation towards the osteoblastic lineage. It is therefore of interest to further investigate signaling molecules, which promote the recruitment, adhesion and activation of critical cell types, including MSCs and osteoblast progenitors. For instance, the temporal expression levels of the pro-osteogenic factors, PDGF-B and BMP-2, were significantly increased from day 1 to day 3 at oxidized implants. This increased expression took place in parallel with an increase in the expression of ALP and OC during the same time period. [75] BMP-2 induces osteogenic differentiation and bone formation by activating the Smad pathway, which in turn induces the expression of Runx2 and other bone-related genes (Figure 1–14). The implant surface properties may further enhance and accelerate the bone formation process at the submolecular level. This assumption was established by the observation that a key osteogenic factor, Runx2, which is a crucial transcription factor for osteogenic differentiation and bone formation, revealed higher expression at oxidized surfaces compared with machined ones on day 3 after implantation, in parallel with the increased expression of osteoblast markers, (ALP) and (OC). [75] Similar results were demonstrated for a hydrofluoric acid (HF) etched

surface in comparison to a similar surface without acid etching. [110] In the latter study, Runx2 expression was evident after 7 days in rat tibia and was associated with a higher expression of ALP and bone sialoprotein (BSP). A regulatory effect on the expression of Runx2 was also confirmed for the HF surface in rabbit cortical bone concurrently with a higher expression of collagen 1 and OC after 8 weeks of implantation. [111] At the implant surface, the collagen-rich matrix organization, together with the increased expression of integrin- β 1, may characterize an important pathway for Runx2 activation and consequently downstream osteogenic activity and bone formation (Figure 1–14).

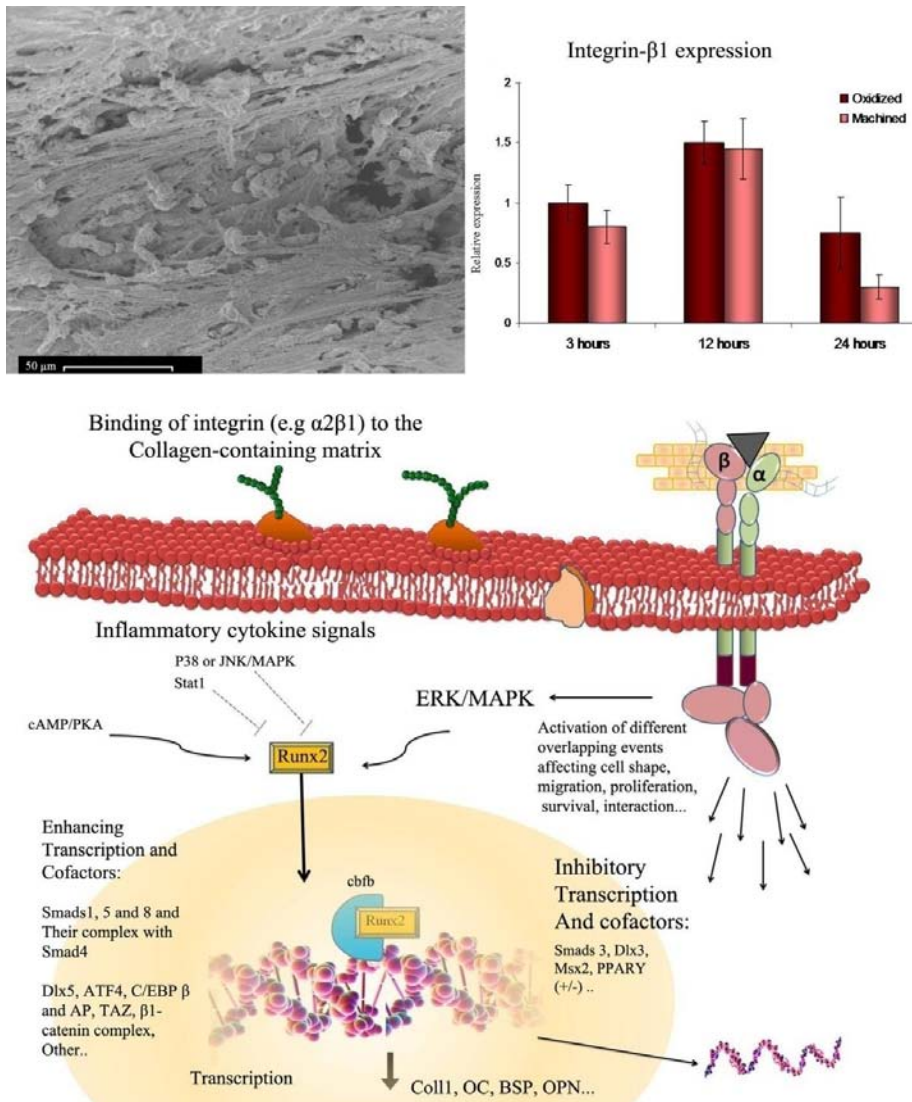


Figure 1.14. Upper right: SEM image of oxidized implants retrieved 6d after implantation. A well-organized collagen network with numerous mesenchymal cells was observed on the oxidized implant. Upper left: a graph showing a significant increase in the expression of integrin- β 1 at an oxidized implant after 1d of implantation. The lower drawing illustrates the activation of osteogenic response by mechanisms involving the binding of integrin- α 2 β 1 to a collagen-rich matrix.

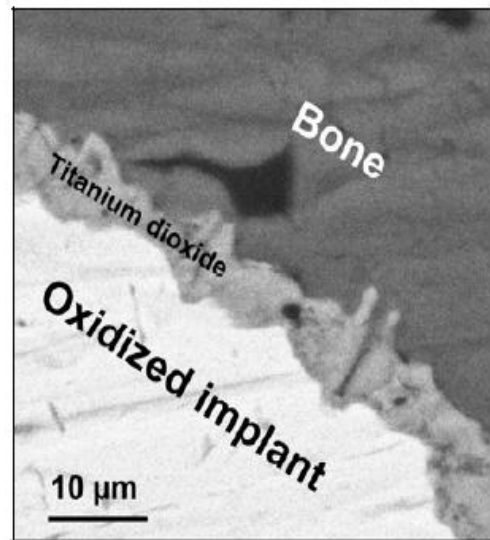
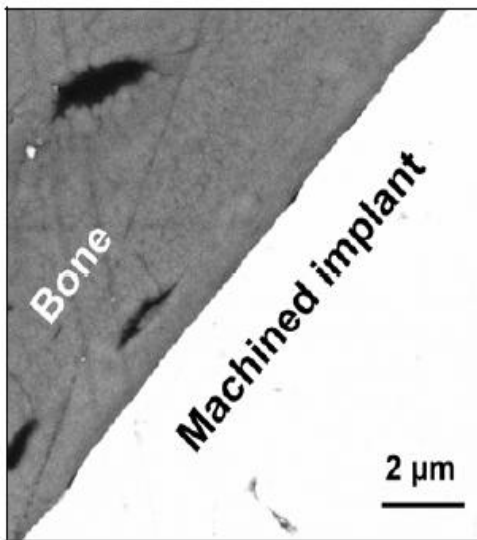
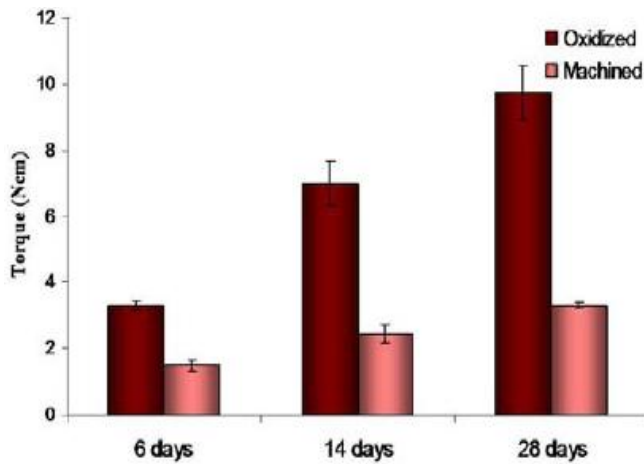


Figure 1–15. Upper: a graph showing a significant increase in the removal torque values for oxidized implants during early osseointegration. The central graphs show the load-deformation curves of machined (left) and oxidized (right) implants after 28d of implantation. The deformation of the machined implant interface is mainly due to separation, whereas a fracture-like pattern is registered for the oxidized implant. The lower backscattered SEM images show a machined implant (left) which was separated from bone by a narrow gap. Direct contact between the bone and the oxidized implant (right) was detected with generalized bone ingrowth into different sized micropores.

Successful osseointegration is best evaluated with respect to the stability of the implant in the implantation site. Studies of gene expression analyses at the interface were therefore further corroborated by removal torsion analysis to determine a possible relationship between the molecular events and torque strength at the interface. [76] A significantly higher and constant increase in removal torque was registered for the oxidized implants throughout the period of 6–28 days of implantation (Figure 1–15). During the same time period, the increased biomechanical strength at the interface of the oxidized implants was associated with higher expression levels of bone formation genes (OC and Runx2) and bone remodeling genes (TRAP and CATK) and the lower expression of pro-inflammatory cytokines. The high

expression of bone formation and bone resorption genes indicated active bone formation and remodeling throughout the time periods, concomitant with the increasing biomechanical strength of the interface. In agreement, other studies compared the expression of different genes and the pull-out force of coin-shaped implants with different hydrofluoric acid modifications after 4 weeks in the rabbit. [112,113] The test implant group that showed increased pull-out values revealed a higher expression of OC, collagen 1 and TRAP and a reduced expression of TNF- α and IL-6.

Low values of removal torque strength were observed for the machined implants during the evaluation periods 6, 14 and 28 days. [76] Machined implants were associated with an increase in the expression of pro-inflammatory cytokines (TNF- α and IL-1 β) during the early (week) phase of osseointegration, [74,75] observations which were also extended to the later stages of osseointegration. [76] It has previously been suggested that the initial reduction and plateauing of the biomechanical strength of machined implants could be a manifestation of post-surgical inflammation and remodeling activity. [114] This is partly supported by these recent data, given that machined implants, which were associated with a higher expression of pro-inflammatory cytokines, did not reveal any increase in biomechanical strength between 6 and 28 days. On the other hand, the increase in remodeling activity coupled with increased bone-forming activity was in line with an increase in interface resistance for similar time periods.

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Part II – Techniques and Methods for Implant Surface Characterization

Chapter II

Surface Topography and Measuring Techniques for Dental Implant Applications – Possibilities and Obstacles

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Abstract

Surface topography has long been identified as a crucial parameter for successful osseointegration of oral implants. A paradigm shift occurred during the 1990s, when the huge majority of companies abandoned turned, minimally rough surfaces in favour of moderately rough surfaces. Several machining techniques have been used to increase the roughness from what was achieved with a turning process. An early obstacle was topographical description of surfaces; scanning electron microscopy (SEM) was often used as a description of the surfaces, but this technique provided no quantitative data. Thus it was, in many cases, difficult to know how one surface differed from another.

Optical methods were developed during the late 1980s, and then it was possible to evaluate the threaded part of the implants relevant for osseointegration. Currently, general guidelines exist for how to measure implants surfaces, and a set of quantitative parameters have, after extensive research, been recommended for the topographical evaluation. However, the implant surfaces of today are often geometrically complex and

include nanometer particles, porosity, coatings and deterministic patterning; thus the demands for new measuring techniques and evaluation methods increase.

2.1. Introduction

Branemark discovered osseointegration—direct bone anchorage—of oral implants during the 1960s. His work led to universal acceptance of oral implants, for the first time ever. Branemark's group [1] advocated the use of c.p. titanium screws with turned surfaces in the bone anchorage portion, i.e., a minimally rough surface according to classification made by Wennerberg and Albrektsson [2]. Our early results had a substantial influence on the more or less abandonment of minimally rough, turned surfaces in the bone anchored portion of the implant. Our results demonstrated an average topography amplitude, S_a , of 1.5 μm and a real-to-projected area ratio S_{dr} , of about 50% to result in a stronger bone response than minimally rough turned or rough, plasma sprayed implant surfaces [3]. These findings resulted in an international shift from minimally rough to moderately rough surfaces.

However, the demands on implant therapy for compromised patients are constantly increasing. New material, machining and coating technologies have offered ways to attack some of these problems. Nanometer structures on an implant surface may have an important role to play in the early healing process around an implant due to their assumed ability to act as adhesion sites for necessary proteins, exemplified by the recent introduction of a coating consisting of about 20 nm sized CaP-particles. The aim behind nanotechnology in implant therapy is to achieve a faster and firmer osseointegration, thus increasing the possibilities for a successful outcome when compromised patients with poor bone quality and quantity are treated with implants.

The surface topography of the soft tissue penetrating part of the implant is smooth (i.e., an S_a value less than 0.5 μm). Although based on empirical findings only, the roughness of abutments has remained largely unchanged to this day. However, nanometer particles may also be of substantial importance for soft tissue adherence and may reduce the risk for infection leading to severe bone resorption. Taking into account previous results from research about optimal roughness at the micrometer level of resolution mentioned above, it is likely that an implant with overall optimal topographical properties includes micrometer *and* nanometer features. To fully understand the biological mechanisms during healing and to be able to design and control the product development of new implants, appropriate techniques for topographical evaluation are necessary.

2.2. Implant Design, Surface Variation on the mm Level

Today, most commercially implants have a screw-shaped design. The introduction of micro-threads was done with purpose of achieving a better stress distribution and better maintenance of the marginal bone height; today many implant companies have implemented this design on their implants based on support from comparative studies by Lee et al. and DeBruyn and Collert (Figure 2–1) [4-5].



Figure 2.1. Micro-threads on the marginal part of an implant are designed to distribute stresses and maintain marginal bone.

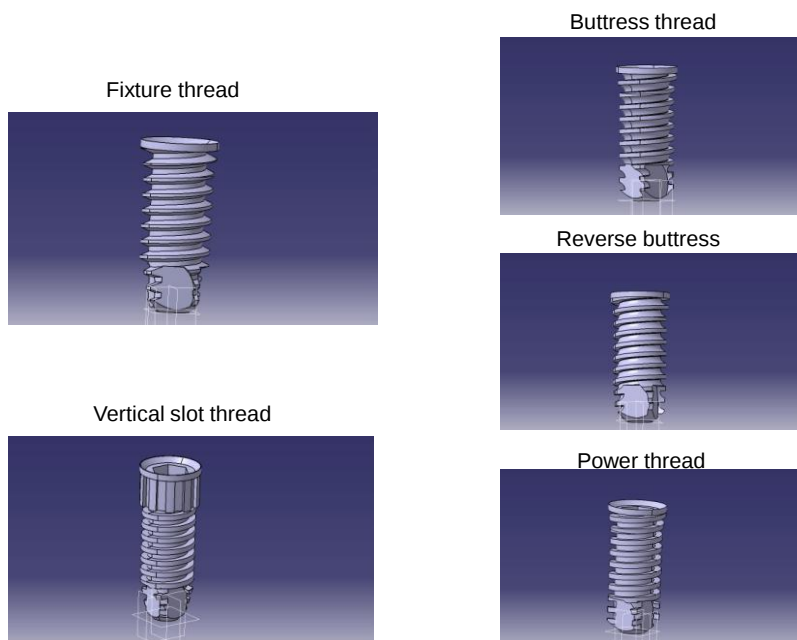


Figure 2.2. Although fixture thread is common thread design, there exist several others; some examples of different thread designs are demonstrated.

Although the huge majority of oral implants are produced with a screw-shaped design, the appearance of the threads may be different. Spiral lock-, buttress-, power-, V-, reverse buttress-, vertical slot-, are just some examples on different thread designs found on oral implants (Figure 2–2).

Independent of thread design, it is impossible to measure all parts of the implant with conventional contact techniques.

The stylus will not have the ability to enter complicated thread patterns and encompass proper reading of narrow threads with high slopes due to geometrical restrictions of the mechanical stylus shape with 90°- or 60° tip slopes and 2, 5 or 10µm tip radii.

2.3. Implant Design, Surface Variation on the µm Level

The machining method for most implants is a turning and/or milling process (Figure 2–3).

To increase the roughness in the µm range, additional machining is necessary. The most common techniques are as follows below.

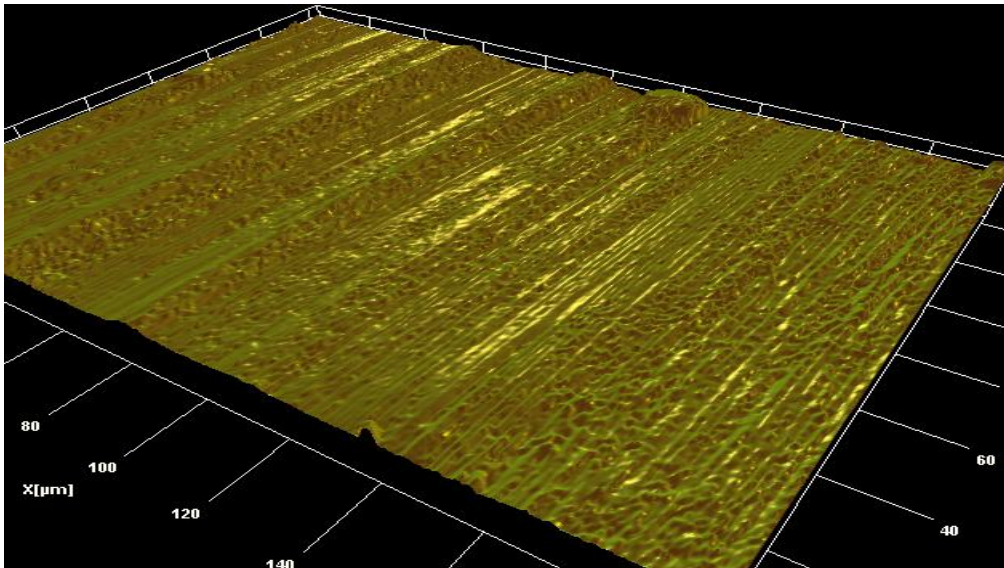


Figure 2.3. A turned implant surface in the µm scale as measured by interferometry. The turned surface has normally a smooth or minimally rough µm-roughness (Here, $S_a=0.53\text{ }\mu\text{m}$).

2.3.1. Increased Roughness by Removal of Material

2.3.1.1. *Blasting*

Abrasive particles of various size and material are shot towards the samples; imprints are created on the surface (Figure 2–4). The created roughness will depend on size, hardness of the material, pressure and distance between the sample and work piece. The chemical composition will be influenced by the blasting material.

The shot blast surface topography is normally characterised by an isotropic texture with height amplitudes and lateral wavelengths determined by the particle size and process parameters used.

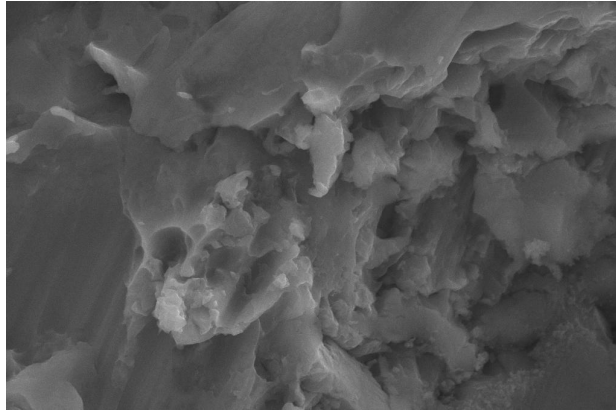


Figure 2.4. A shot blasted dental implant. The micro roughness has been increased by the imprints of the blasting particles hitting the surface. A SEM image, X 50 000 magnification.

2.3.1.2. Oxidizing

Anodic oxidation is performed with the titanium sample positioned as the anode. The roughness will depend on the selected electrolytes, current and voltage. The chemical composition may be altered depending on the content of the electrolytes.

The anodically oxidised surface topography is characterised by a porous "crater" texture with an isotropic distribution of various circular crater diameters at various heights, depending of the shape of the sub- μm thick Ti-oxide layer. The thin oxide layer is super positioned on the underlying machined implant surface, and the combination of oxide layer and pre-machining determine the joint height amplitudes and lateral wavelengths (Figure 2–5).

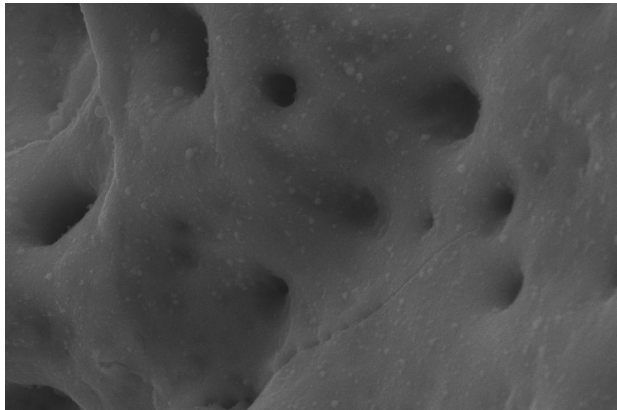


Figure 2.5. An anodic oxidised dental implant. The crater-formed micro roughness is a result of the electrical currents altering the naturally smooth Ti-oxide surface to a "moon-like" crater landscape. An SEM image, X 50 000 magnification.

2.3.1.3. Etching

Although titanium is known to be very resistant towards corrosion, still some acids can be used to alter the topography. Etching often does not increase the roughness but changes the anisotropy of milled and turned surfaces to isotropic surfaces. Further, the technique will remove some sharp peaks and add high frequency irregularities. HCl and H_2SO_4 are often

used acids for etching oral implants. The chemically etched surface topography is characterised by a structure consisting of steep-walled pits or irregularities distributed over the surface in a more or less regular pattern, depending on Ti-grain size and grain distribution in combination with etching process variables like chemical composition, etching time and temperature. Height amplitudes and lateral wavelengths are determined by the size and distribution of the pits or irregularities (Figure 2–6).

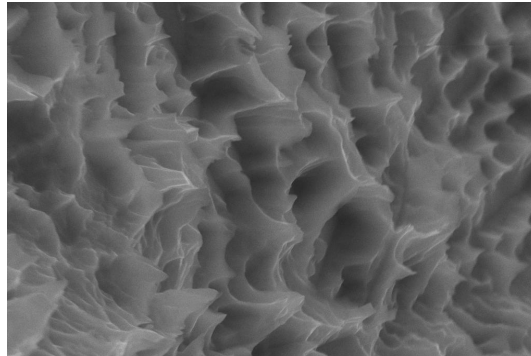


Figure 2.6. An etched dental implant. The micro roughness is a result of the chemical erosion of the Ti-oxide, -grains and -grain boundaries. An SEM image, X 50 000 magnification.

2.3.1.4. Blasted and Etched

A combination of blasting and etching has become quite common. To combine these techniques will result in a moderately rough surface (based on the shot blast texture) with certain wavelengths and superimposed high-frequency components (the latter mainly a result of the etching process) (Figure 2–7).

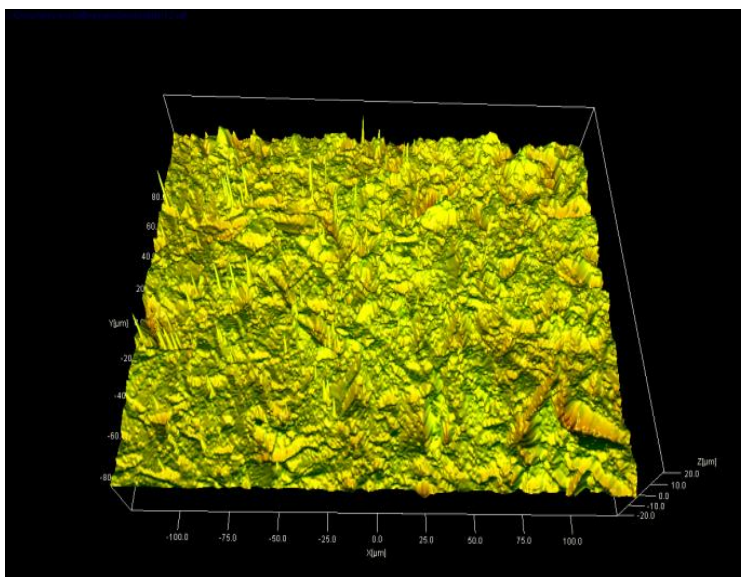


Figure 2.7. A shot-blasted and etched dental implant. The micro roughness is a result of the combination of the isotropic blast structure super imposed by a high-frequency chemical etch pitting and a chemically "smoothened" blasting texture. The image is produced from data achieved from interferometry.

Etching a blasted surface will often result in a slightly decreased height deviation due to the (chemical) removal of very sharp and tiny peaks.

2.3.2. Increased Roughness by Adding Material

Titanium plasma spraying (TPS) and hydroxylapatite (HA) μm coating have been used to increase the roughness and alter implant chemistry. Both techniques add material to the surface, but neither of them is commonly used today.

2.4. Implant Surface at the nm Level. Common Manufacturing Techniques

2.4.1. Nanometer Coats

Very thin coats or rather droplets of CaP, HA (hydroxyapatite) or TiO_2 on the implant surface are found on some commercially available implants.

The aim with an alteration of the surface topography at the nm level is to enhance bone formation through chemistry by creating adhesion sites for proteins that will rapidly cover the implant surface after insertion.

The nanometer coated surface topography is characterised by a structure consisting of steep-walled "droplets" distributed over the surface in a more or less regular pattern (Figure 2–8).

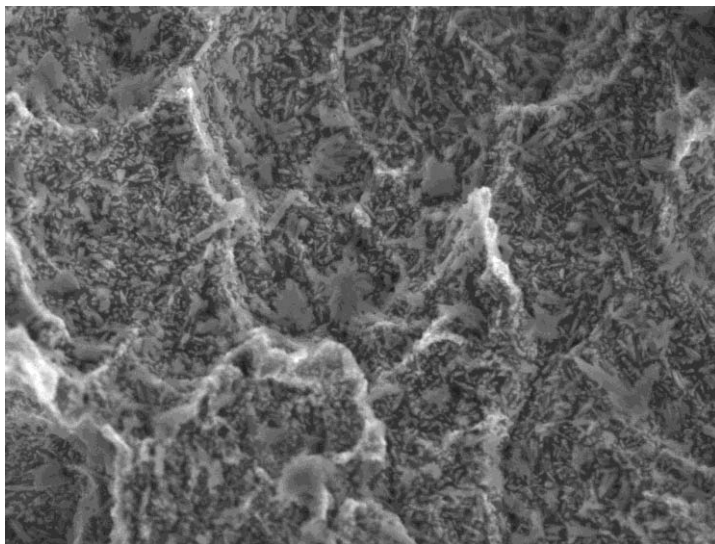


Figure 2.8. Nanometer "droplet" coated topographies of dental implants. The coating process will result in specific nano structures. An SEM image, X 30 000 magnification.

2.4.2. Ion Incorporation

Calcium, Magnesium and Fluoride are examples of ions that in experimental studies have demonstrated a positive effect on bone healing. These chemical modifications alter the nm topography, too.

2.4.3. Hydrophilicity

Increasing the wettability by cleaning under nitrogen protection has been suggested to have an impact on bone formation, even if experimental in vitro and in vivo studies lacked control of all surface parameters [6-7].

2.5. State-of-the-Art Metrology

The task of surface metrology in the field of characterizing biomedical materials is demanding, since the physical and chemical environments are more complex than in the realm of conventional materials. The dimensional range of scale of such surface texture and topography measurements is from molecules to proteins (nanometric) to cells (micrometric), with the critical parameters being size, shape distribution of features (which can be discrete—holes or peaks—or continuous, such as ridges and furrows) and whether random or periodic. A multi-scale approach to measuring some surfaces may be useful [8].

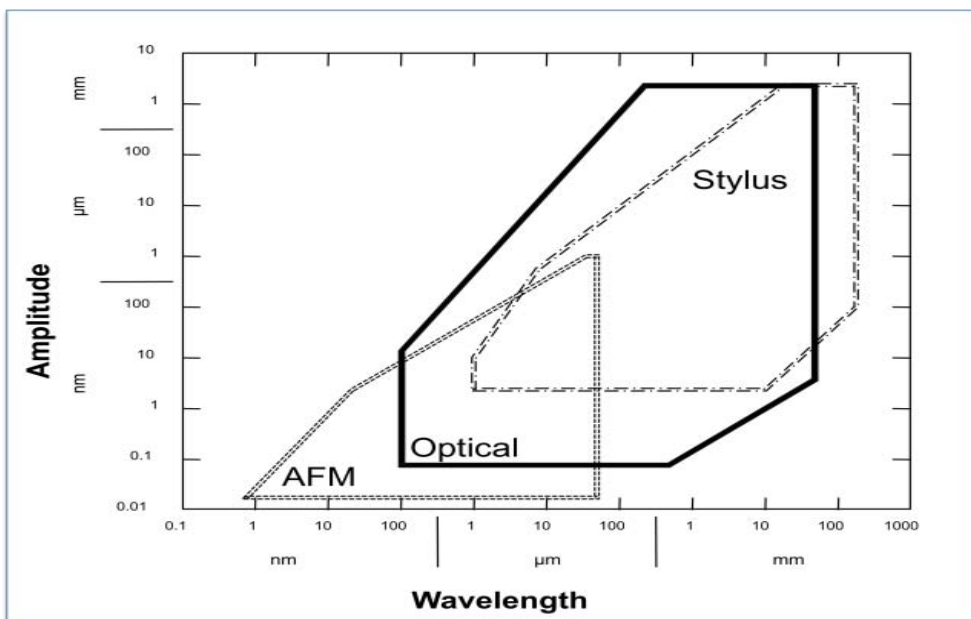


Figure 2.9. The Amplitude-wavelength space diagram -Steadman diagram, describing the operating areas covered by the optical and scanning probe (AFM) and contacting stylus instrument families.

The development of metrology instrumentations for surface topography has been extensive, especially for non-contact optical and scanning probe instrumentations. The multi-scale characteristic of implant surfaces highlights the importance of selection of instrumentations covering the appropriate vertical and horizontal scales. The Steadman diagram is an illustrative way of illustrating the operating regimes for different instrument types (Figure 2–9).

2.6. Measuring Methods. Instruments

2.6.1. Contact Profiling Methods

The characteristics, advantages, shortcomings and fields of application of contacting instruments are well known. The advantage of contact profiling methods is their robustness and use for many decades in mechanical industry as a standard technique; thus there exists a lot of experience. Furthermore, several standards are written to suit the contacting mode.

The disadvantages include the risk of surface deformation due to the pressure of the contact tip as a result of the standardised (ISO 3274) 0.75mN measuring force, the limited access of the stylus into small samples due to the styli angle of 60° or 90° and the styli limited resolving of finer details due to the 2-10 µm radius. Another limitation is the measuring speed, which for most contacting instruments is between 0.3-1mm/s.

Applications

Contacting instruments have a large dynamic range from nm to several mm, i.e., where the range and resolution of vertical height measurements enables the texture information to be measured simultaneously with form. Millimeter-scale profiles of the treads of dental implants can be assessed if the stylus shape does not limit the access due to slopes of thread flanks and/or narrow thread bottoms. Contacting techniques are especially advantageous where very steep-sided (<60°) features need to be evaluated [9-10] for the variety of probes that can be used.

2.6.2. Non-Contacting Optical Methods

Light waves of different wavelengths interacting with surfaces have been used extensively for measurement of dental implants for many years. The main advantage with optical techniques is the non-contacting measurements along with decreased measurement time compared to the contact profiling methods. There exist a multitude of optical techniques. One classification of their properties is to divide the techniques into Scanning- Areal-, Scattering optical instruments and Scanning Electron instruments, the latter more for non-quantitative imaging.

The limitations of the optical instrumentations using microscope objectives are *firstly* the largest slope angle possible to measure is restricted by the numerical aperture (NA) of the objective used. The NA determines the highest slope on the surface still enabling the light to be reflected back into the objective.

Table 2.1. Examples of the lateral resolutions and maximum slopes for a set of microscope objectives used in air with refractive index $n=1$ and a light wavelength λ of 570nm

		Magnification					
		2.5x	5x	10x	20x	50x	100x
Objective data	Numerical aperture, NA	0,075	0,13	0,3	0,4	0,55	0,8
	Working distance (mm)	10,3	9,3	7,4	4,7	3,4	0,55
	Field of view, FOW (mm*mm)	2,81* 2,10	1,47*1 ,05	0,70*0 ,53	0,35*0 ,26	0,14*0 ,11	0,07*0 ,05
Lateral Optical resolution, $\lambda=570\text{nm}$	"Rayleigh" (um)	4,6	2,7	1,2	0,9	0,6	0,4
	"640x480 pixel spacing" (um)	4,4	2,2	1,1	0,5	0,2	0,1
	"1145x860 pixel spacing" (um)	2,5	1,2	0,6	0,3	0,1	0,1

Secondly, the horizontal resolution is limited by the largest resolution estimated by the Rayleigh or Sparrow criterions or the distance between individual pixels in the measured image (Table 2–1). *Thirdly*, surfaces consisting of materials with varying refractive and adsorption indexes can give significant phase changes, i.e., significant modification of the true surface geometry, a factor which has to be checked in the individual surface measurement.

For scanning optical techniques, the size of the scanning light spot (often in the size of 1-2um diameter) puts restrictions to the lateral resolution possible since the light response from the surface will be averaged over to spot size.

Many different types of optical microscopy techniques have been successfully used to measure surface texture, scanning or phase shifting interferometry as well as confocal microscopy [11-13]. Advantages are speed of operation and lack of surface damage and high lateral resolution (compared to traditional mechanical styluses) and represent very important factors when choosing the optimal probing technology. The major disadvantage of the technique is the need for a reflecting capacity from the sample to be measured; if this is too low, invalid data will be present for reliable evaluation.

Applications

Interferometry has been proven to be a suitable technique for the huge majority of oral implants [2, 14].

Phase-Shifting Interferometry

Phase shifting interferometry (PSI) is based on single wavelength light illuminating a surface through an "interferometer" objective consisting of focusing lenses and semi-transparent beam splitter where one wavefront is reflected on the surface to be measured and the other reflected on a reference surface. Both reflected wavefronts are projected to a common CCD array and result in a series of interference patterns varying as the distance to the surface is shifted by a piezo electric vertical scanning stage.

By combining three or more images of interference patterns within a height scan less than one wavelength of the used light, the intensity of the individual pixels at the different heights

can be combined to calculate the height at each x- and y- coordinate, resulting in a 3D height map.

The main limitation of the technique is that surfaces with peak-to-valley distances larger than half the wavelength of the used light (typically around 500nm) will not be detected. The main advantages of the PSI technology are the sub-nanometer vertical resolution and the fast repeatable measurements possible.

Coherence Scanning Interferometry

Coherence scanning interferometry (CSI), or white light scanning or vertical scanning interferometry, is a way to overcome the shortcomings of the PSI by using a low coherence broadband white light illumination and, for each individual pixel, to determine the phase (intensity) while the vertical piezo actuator scans the interference fringes through the total peak-to-valley distance of the surface. The vertical position for the individual pixel is in the CCD array, where the contrast of the fringes maximum is determined and is combined with the other pixels to form the total 3D height scan.

The vertical range is typically around 100 μm but can be increased by replacing the vertical piezo actuator with a long-range (several mm) translator. The lateral resolution is limited by the largest field of view (FOW) and the pixel amount in the CCD array used or the Rayleigh or Sparrow criteria.

2.6.3. Confocal Instruments

The lateral resolution in a conventional light microscope can be increased by reducing FOW e.g., using drilled "pin- holes." The combination of the reduced FOW with a controlled vertical focus scan results in an intensity maxima when the pin-hole image is in focus, thus enabling the height coordinate of the vertical focus scan establishing the height coordinate of the individual pin-hole image. By scanning the surface using rotating discs with several drilled pin-holes under the detector, the focused individual "pin-hole" height coordinates construct a 3D mapping in x,y and z-coordinates of the surface. The vertical resolution may be of a few nanometres, and horizontally, the resolution is similar to other optical instruments in the range from 0.3 μm to 2 μm .

2.6.4. Scanning Electron Microscopy

Scanning electron microscope (SEM) images the sample surface by scanning it with a high-energy beam of electrons in a rectangular pattern. The electrons interact with the surface atoms within a volume determined by the energy level of the beam and surface material and produce signals with information about topography and material properties such as types of atoms for an elemental analysis. The types of signals produced by SEM include secondary electrons, backscattered electrons (BSE), and x-rays. SEM produces high-resolution images of a surface, resolving details about less than 1 to 5nm in size. SEM micrographs have a large depth of field creating a characteristic three-dimensional appearance useful for understanding the surface structure of the sample. A wide range of magnifications are possible, from about

ten times to more than 500,000 times. The resolution of the SEM is not high enough to image individual atoms, as is possible in the shorter wavelength (i.e., higher energy) transmission electron microscope (TEM). Three-dimensional data can be measured in the SEM, e.g., by using images of the surface taken at different angles or and decomposed into 3D. One further development is environmental SEM (ESEM), a technique where coating of non-conductive materials are avoided and samples emitting vapor-like wet biological samples may be imaged and x-ray analyzed without the disturbing coating. In the ESEM, the conventional SEM vacuum is replaced by a pressurized gas and an electrically charge by the electron beam surface is avoided.

2.6.5. Scanning Probe Microscopy

This family includes scanning tunneling microscopy, (STM) (tunneling current contrast) and atomic force microscopy (AFM) (interatomic force contrast) [15].

In the STM, a single atom "sharp" tip of a conducting material is positioned within nanometers from the surface to be measured. When a small voltage is applied between the tip and measurand, electrons will move (tunnel) across the gap and realize a measurable current related to the distance between tip and surface, hence a signal relative to topography changes while the tip is scanned across the surface. The sensitivity of the system allows for down to $0.01\text{\AA}$ vertical resolution. The lateral resolution is normally limited by piezo drive and in the range of $1\text{\AA}$.

The AFM overcomes the STM limitations to conductive surfaces by typically using a Si or SiN stylus with an ultra-fine tip of about 10nm diameter. The tip is traversed over the surface either in close contact with the interatomic repulsive surface forces (near field measurements) or by attractive Van der Waals or capillary forces (far field measurements). A measurement system is commonly based on measurements of the tip cantilever deflection during the x-y traverse by detection of a light beam reflected on the deflecting cantilever on a light-positioning sensitive surface some distance away, producing a signal relative to the changes of surface topography beneath the tip and a combination with the x-y position produce a 3D topography map of the surface.

The AFM measurements may be disturbed by surface contamination, by trapped electrostatic charge and, in atmospheric measurements, by very commonly adsorbed 10-30 monolayers thick moisture influencing the tip-surface forces.

Martin et al. [16] introduced a non-contact technique where the tip is oscillated and amplitude of oscillations modulated by the weak attractive Van der Waals forces. Problems that occur with the interaction of the adsorbed fluid layers when using the noncontact technique led to the development of the "tapping" mode scanning where the tip is oscillated at a frequency close to the resonant frequency, and, while moved towards the sample, the amplitude of the tip oscillation reduces. The "tapping" significantly reduces the problems of electrostatic forces and moisture.

In practice, AFM [17-20] has the advantage working in the presence of hydration or even liquid water in vivo. The AFM stylii with a nanometer sized sensing tip will enable Ångström 10^{-10} m resolution in three dimensions. One of the limitations with this technique is that measurements are restricted to conductive surfaces. Scanning sizes are typically about $100\mu\text{m}$

laterally and 5µm vertically, making the technique unsuitable to evaluate several surface modifications found on modern oral implants.

Applications

The AFM is the measurement device candidate for the high-resolution measurements needed to quantify and image nano-sized HA features.

2.7. Characterisation

The aim with surface topography characterisations is to find unique numerical parameters or functions describing geometrical features of the implant surface [21]. The meaning of unique in this sentence is parameters relevant to the function or manufacturing of the implant.

An engineering surface is a geometrical combination of overlay features in different scales. Overall form (plane, cylinder...), waviness (machining vibration, feedmarks) and the finer nm- or um-large structure consists of peaks, valleys, scratches, pits, ridges, porosity, etc. The first decision will be if a 2D or 3D characterisation shall be done.

Two-dimensional descriptions can be used to describe relatively simple surfaces and simple tread geometries, but very often a three- dimensional (3D) representation of a surface is required to quantify directional surface features like "craters," "droplets," pits, smoothen peaks, etc.; thus this largely excludes 2D metrology and 2D characterisation from being employed for dental implant.

Procedures in characterisation is a sequence of activities including measuring, segmentation and quantification. The measuring has to be based on a sampling strategy.

2.7.1. Sampling Strategy

The possibilities for surface geometry characterisation using 3D metrology [ISO25178-2:2007] range from robust statistical parameters like the average height deviation (S_a) or its surface-related parameter, Root-Mean-Square ($RMS = S_q$), to more specialised summit shape and texture directions descriptors. However, basic metrology strategy issues like:

- resolution,
- sampling size,
- sampling area positions,

still need to be clarified for each measurement task. Implant surface regions vary considerably in roughness, and it is advantageous to sample each region independently. Stratification is the process of grouping the whole surface area into relatively homogeneous regions before sampling.

The top of the dental implant treads have been reported to be considerably rougher than the flanks or tread bottoms and can vary several hundreds of a percent [2,3,14]. Tailor-made sampling schemes are here necessary tools to decrease the uncertainty of measured textures [22]. The positioning of the sampling areas on the implant surface need to be carefully

considered. Too few measurements or measurements placed at non-representative positions may cause erroneous decisions and unnecessary costs. Here, investigations regarding number of samplings and positions need to be undertaken for each dental implant type. For turned and blasted implants, an averaging of a set of 3+3+3 measurements on the flank, the top, and in the bottom of the thread results in reasonably stable means of chosen numerical parameters. The following segmentation is the separation of the measuring data up into functional wavelengths or features at given scales.

2.7.2. Segmentation

In order to analyse a dental implant surface, a common method is to decompose the surface into bands of wavelengths using digital filters. The digital filters have defined limits for the shortest and the longest wavelengths to extract from the surface measurement. The most commonly used filter for the separation of surface wavelength is the "Gaussian filter," defined in ISO 11562:1996 for 2D profiles. In ISO 3D terms, this is called studies of scale limited surfaces, and the two limiting wavelengths are named nesting index at the scales S and L. Quantification is the process where the segmented data is translated into selected statistical or deterministic descriptions of the functional features, e.g., mean amplitude, mean wavelength and number of pores and valley volume.

2.7.3. Quantification of Surfaces

Surface geometry may be quantitatively described using 2D profiles or the real 3D surface as a basis for calculations. Before the mid-1990s, the common practice for dental implant surface geometry specification were at best the 2D profile amplitude parameters R_a and R_t describing the average and maximum amplitudes of the profile (ISO 4287:1996). Extensive research in the area by Wennerberg and others [2] resulted in the establishment of a parameter set for dental implant characterisation based on the currently EUR 15178N and ISO 25178 standards.

2.7.4. Field Parameters

The Field parameters are parameters describing the average, the extreme, and other statistical properties of the wavelength segmented or scale-limited surface like Average amplitudes or Average slope angles, Total height, Core void volume, Peak heights.

There are 12 field parameters defined in the standard, and they are divided into five groups: Height parameters, Spatial parameters, Hybrid parameters, Bearing area-based parameters and Fractal-based parameters. Many of the parameters in the Amplitude, Spatial and Hybride groups are well known from the 2D profile world with P-, R-, or W- instead of the S letter used here. S means surface, and, for example, the height parameter describing the surfaces root mean square height (of the scale limited surface) is noted S_q where the q is a

suffix specifying the meaning of the specific S parameter (here, q refers to the q in root mean square height expression).

The Bearing area-based parameters use a mix of the V-letter before their suffixes. The V stands for Volume and is used for both material and void volume descriptions. The Bearing area parameters like Pit Void Volume (V_{vv}) give a useful description of the volume of implant surface porosity below given material to void ratio, p, with a possible range from 0-100% material. All the V-parameters show interesting possibilities of the material and void properties at different heights of the surfaces.

2.7.4.1. Height parameters

The Height parameters describe the surface deviation from an intersecting mean plane. Sq and Sa describe average height amplitudes either by the RMS method or as a simple arithmetic mean.

The shape of the amplitude distribution curve is quantified by its skewness (S_{sk}) and the kurtosis (S_{ku}). Maximum peak and valley points are described by S_p and S_v, and their sum is named maximum height, S_z.

The average amplitude parameters are very well suited for a robust characterisation of the overall roughness of dental implants. The parameters are especially well equipped for control of isotropic implant surfaces like shot blasted or amplitude information dominated screw surfaces like turned ones, where no detailed surface feature information is required. The extreme amplitude parameters S_p, S_v, S_z are normally very sensitive to noise or spikes in insufficiently segmented data and less stable for surface descriptions.

2.7.4.2. Spatial Parameters

The Spatial parameters describe the lateral properties of the surface. Auto correlation (S_{al}) indicate the starting wavelength of repeating features, while the presence of anisotropic properties is shown by the texture aspect ratio (S_{tr}), defined as a quotient between the shortest and longest autocorrelation lengths in any directions of the surface. The texture direction parameter (S_{tr}) is in ISO grouped as a "miscellaneous" parameter but defines the direction of the largest autocorrelation length. The spatial S-parameters are excellent for detection of anisotropy of underlying structures of the dental surface that have not been removed in previous manufacturing steps.

2.7.4.3. Hybrid Parameters

Hybrid parameters describe the shape of the surface by the mean slope (S_{dq}) and the developed area ratio (S_{dr}), i.e., by a combination of amplitude and spatial properties. The latter is a measure of the total surface area compared to a nominal flat area.

The Hybrid parameters have a strong potential to give numbers, useful for, e.g., characterisation of active surface area (S_{dr}). The hybrid parameters are very scale sensitive and must be measured at a scale where functional wavelengths are present.

2.7.4.4. Bearing Area Curve-based Parameters

The Bearing curve describes how much material and how much air the surface contains at each level from the highest peak to the lowest pit in the surface. The function is implemented

by the intersection at different levels of the surface by planes parallel to the mean plane (see Figure 2–10).

The parameters based on the bearing curve (Sk Spk, Svk, Smr1, Smr2) are tailored to seek the peak, core and valley regions of the surface, based on the assumption of a negatively skewed surface (Ssk negative, indicating a valley biased surface).

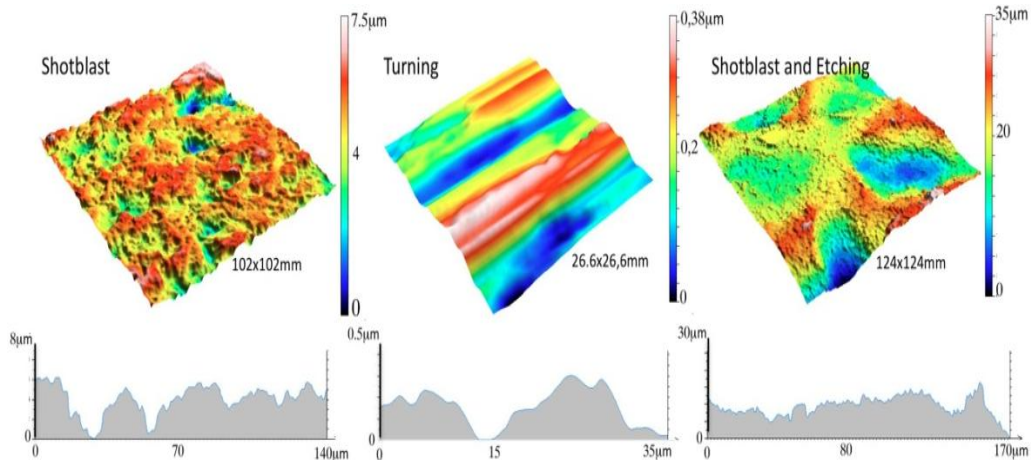


Figure 2.10. The traditional 2D surface profile contains general statistical amplitude and spatial wavelength information, but the characteristic properties of the 3D topography of modern implant surface features like valleys, "craters," "droplets," pits, smoothed peaks, etc., need 3D representation for a proper characterisation. 2D profiles are extracted in the "top-bottom diagonal."

The areal material ratio parameters (Smr) and (Smc) indicate the material ratio or amplitude at a predefined height (c) or ratio (p).

The bearing area parameters are mostly suited for tribological application with surfaces having distinct plateaus and valleys, but also porous and etched implant surfaces show similar plateau like geometry and should therefore be suitable for bearing area evaluations.

Conclusion

Implant topography at the micrometer level of resolution has been found strongly correlated to the bone response that shows a peak to moderately rough surface characterized by an Sa of 1-2 µm. From a clinical perspective, it seems like ordinary bone sites can tolerate a relatively wide spectrum of implant surface topographies, but compromised bone sites display significantly better clinical results for moderately rough surfaces compared to smoother or rougher ones. There is clear evidence that nanoroughness and implant chemistry may influence the bone response as well, but here we lack clinical evidence of their efficacy, and it is, at present, impossible to identify the "ideal nanotopography." Chemically altered surfaces supported by, e.g., calcium, magnesium or fluoride ions have been clinically documented with excellent outcome, but we still lack clinical evidence from comparative, controlled studies that so-treated surfaces outperform non-treated specimens. Surfaces with physically altered conditions, such as high surface energy-treated implants, lack clear experimental evidence of them resulting in an improved bone response, since performed

studies have failed in controlling implant topography that just as likely may explain the experimental findings as the high-energy state. In essence, implant surfaces may have a strong impact on the osseointegration response, and it seems likely that we will learn how to present more clinically ideal surfaces in the future.

A wide range of surface features on modern oral implants requires a careful strategy for topographical characterisation. Instruments, measuring areas, number of measurements and parameter selection are all important factors for a reliable outcome.

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Chapter III

Surface Properties and Characterization Methods of Titanium Dental Implants: Topography, Chemistry, and Oxide Thickness

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Abstract

Implant surfaces have long been recognized as playing an important role in successful osseointegration. Unlike over the past three decades, recently developed micro- and nanotechnology is rapidly advancing surface engineering in implant dentistry. Such advances in surface engineering technologies have resulted in more complicated surface properties at the micro- and nanometer scales, including morphology, chemistry, crystal structure, physical, and mechanical properties. Hence, the well-defined characterization of the implant surface is fundamental to understanding the exact roles of implant surface properties on osseointegration phenomena and for the further development of smart implants. This chapter will focus on surface characterization techniques that have been more commonly used to probe the surface structure, chemistry, and oxide thickness of clinically available titanium dental implants. The characterization methods include scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS or *EDX*), Auger electron spectroscopy (AES), and X-ray photoelectron spectroscopy (XPS). The basic principles that underlie these techniques are described to better understand their applications to surface characterization. The surface characterization methods are exemplified by analyzing clinically available titanium dental implants, including Tioblast[®], Osseotite[®], Osseospeed[®], SLA[®], SLActive[®], TiUnite[®], Ospol[®] and Implant M[®], resulting in a variety of surface properties because of the differences of the surface engineering techniques used in the applications of each

implant. Recent advances in the surface engineering of titanium dental implants necessitate comprehensive surface characterizations at the micro- and nanoscale.

3.1. Introduction

Today, the majority of dental implants in clinical use are made of titanium or titanium alloy. Surfaces of titanium dental implants have long been acknowledged to play an important role in osseointegration performance. A number of publications have been dedicated to surface characterization methods concerning, generally, the titanium biomaterials, with many of the applications related to properly prepared “flat” samples. The interested reader is referred to many useful books on surface analysis methods [1-5]. However, the situation can differ for the dental implants, of which almost all are in screw shapes, which sometimes leads to difficulties during direct application of general instrumentation using analytical equipment. This chapter deals with the characterization of titanium surfaces (oxides) of screw-shaped dental implants.

To improve the clinical success of titanium dental implants, modern surface engineering techniques have expanded to include new and novel implants with complicated surface properties at the micro- and nanoscale. Comprehensive surface characterization of the implants provides fundamental information about the surface properties of the implants, which advances our understanding of *in vitro* and *in vivo* performance of the surface properties, thus guiding appropriate suggestions for the development of smart implant surfaces. A variety of techniques for surface characterization are applicable to titanium dental implants. However, there is no guideline or consensus about what surface properties of dental implants must be evaluated and what surface characterization method(s) should be undertaken for them.

Current innovations in surface chemistry combined with new trends to apply surface nanotechnology to titanium dental implants demand further details that characterize the implant surface. Poor characterization may result in misleading perceptions about the roles of the surface properties in implant performance. Another reason of great importance to understand the surface properties and their characterization methods is that findings show that control over the surface quality can make a distinction between the roles of surface chemistry and topography that differs for different bone healing stages. Thus, we can validate at least the underlying mechanisms of osseointegration, i.e., the biochemical bonding and/or the mechanical integration at the implant/bone interface. [6-9]

Of the many surface parameters of dental implants, the surface structure, chemistry, crystal structure, and roughness have been recognized as key parameters that enhance the tissue response to implants and therefore have often been evaluated (Figure 3–1). This chapter will focus on surface characterization methods related to the surface structure, the chemistry, and the oxide thickness of titanium dental implants, including scanning electron microscopy (SEM), X-ray spectroscopy (EDS or EDX), Auger electron spectroscopy (AES), and X-ray photoelectron spectroscopy (XPS). The other methods will be covered in another chapter of the book. For a surface characterization, an operational definition of “the surface region” or “surface” is the volume of a solid that a measurement technique samples (the Auger Technical Instruction Workbook. Perkin Elmer Corporation, Physical Electronics, 1990). The

units of measurement in this chapter are the following: $1\text{ mm} = 10^3\text{ }\mu\text{m}$, $1\text{ }\mu\text{m} = 10^3\text{ nm}$, $1\text{ nm} = 10\text{ }\text{\AA}$, and $1\text{ }\text{\AA} =$ approximately the diameter of a single hydrogen atom.

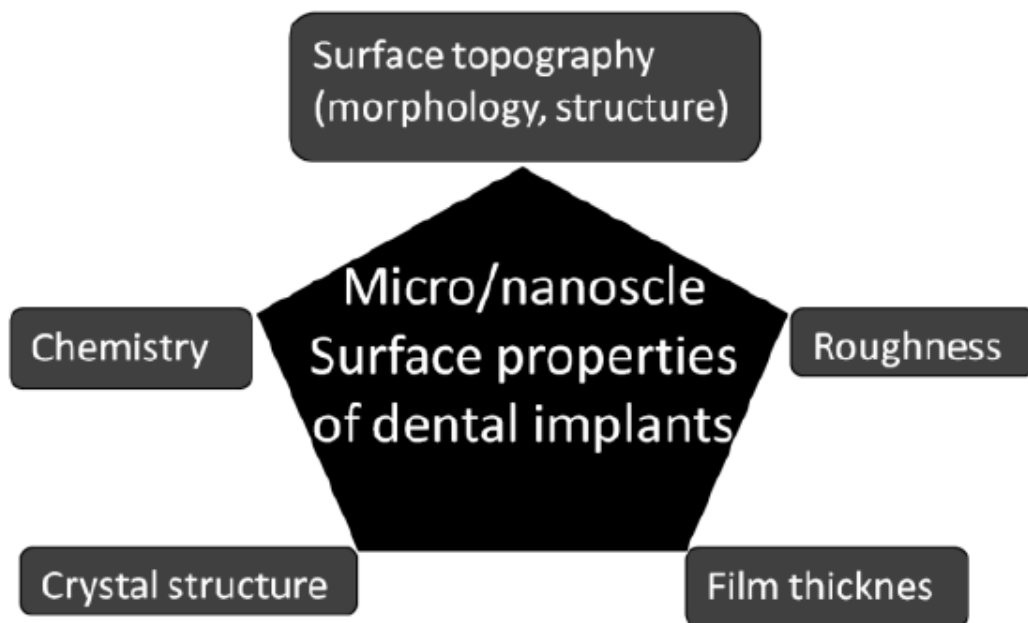


Figure 3–1. Major surface properties of titanium dental implants that contribute to the improvement of osseointegration.

For presenting a variety of the surface characteristics of titanium implants, surface characterization methods were performed on a number of clinically available titanium dental implants, including machine-turned implants, Tioblast[®], Osseotite[®] Osseospeed[®], SLA[®], SLActive[®], TiUnite[®], Ospol[®], and Implant M[®], which utilize applications of various surface engineering techniques.

3.2. Surface Properties of Titanium Dental Implants

The surface oxide property of titanium and titanium alloy implants plays a vital role in successful osseointegration. Surfaces of machine-turned titanium implants are covered with a thin, cohesive oxide film that is spontaneously formed in contact with air, which primarily determines the surface properties of the implants. Excellent biocompatibility of titanium implants (surface oxide) has been reported with respect to various properties, such as a low level of electronic conductivity [10], a high corrosion resistance and a thermodynamically stable state at physiological pH values [11-13], and a low ion-formation tendency in aqueous environments [14].

However, the native oxide film is very thin and hardly measurable with the direct method of SEM observation on a cross-section, and the film is 1.8 - 17 nm thick, as indirectly measured by sputtering methods in AES and XPS. The chemical composition of the native

oxide film consists mainly of TiO_2 [14-19]. The oxide structure of the native oxide was found to be noncrystalline but tends to be crystallized, as shown by the peak intensities at 136 cm^{-1} , 140 cm^{-1} , measured by Raman spectroscopy [19]. The surface textures of machine-turned titanium implants showed grooves $\leq 10\text{ }\mu\text{m}$, occasional pits and protrusions, and a wavelength $\approx 30 - 50\text{ }\mu\text{m}$.

To improve the clinical performance of the machine-turned titanium implants, several methods of topographical modification such as etching, blasting or hybridization have been widely applied over the last three decades and have produced microstructured implants with moderately rough surfaces varying $0.7 - 1.4\text{ }\mu\text{m}$ in the S_a value. Whatever the topographical modification methods are used, the surface chemistry of the implants consisted of TiO_2 , and crystal structures presented amorphous phases although there are fine differences in the titanium oxide stoichiometry (see the XPS section) [20-22].

Recently, emerging nanotechnology in implant dentistry has made rapid advances in surface chemistry modification, which is exemplified by the clinically available surfaces of Osseospeed[®], Nanotite[®], SLActive[®], TiUnite[®], Ospol[®] and Implant M[®]. (Each property of the surfaces will be presented in the corresponding section below.) This advance provides a new era of challenges and opportunities in surface research. A better understanding of characterization methods and resultant surface properties may benefit from advancing our knowledge about implant surfaces and their roles in osseointegration. Surface properties of some of the screw-shaped titanium dental implants using the micro- and/or nanotechnologies are summarized in Table 3–1.

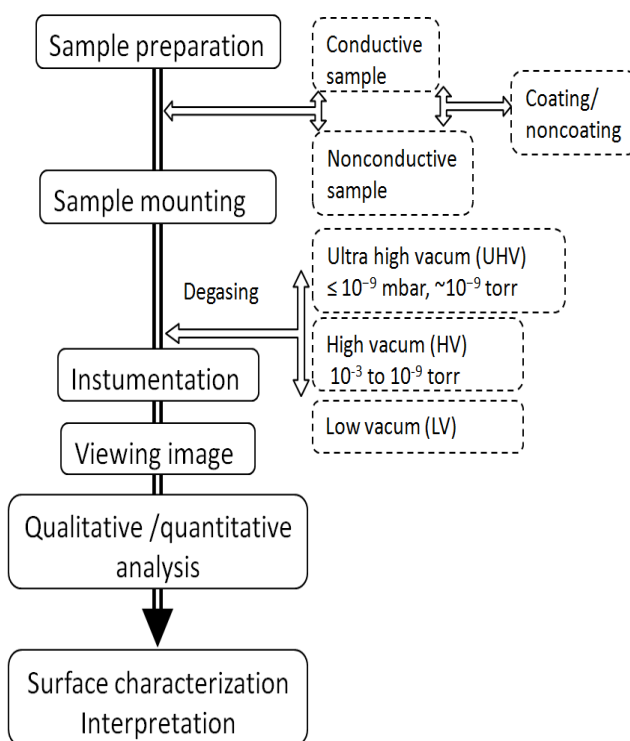


Figure 3–2. General procedure of sample analysis for surface characterization in vacuum equipment (For details, see text).

3.3. Surface Characterization Methods

3.3.1. Sample Preparation

Figure 3–2 illustrates the general procedure of sample analysis performed in vacuum equipment. First, proper sample preparation is a crucial first step towards obtaining a high quality of surface characterization. For surface characterization of the samples in vacuum equipment, sample coating is very common. A major reason for the coating is to provide conductivity for a non-conductive or less-conductive layer, thus more effectively obtaining signals of the electrons, X-rays and/or photons that are emitted from the surface region of the samples. Sample preparation for surface characterization is very important to survey exact surface properties of the implant samples. A detailed review of sample preparation methods, however, is beyond the scope of this chapter. Sample preparation is briefly described in each section of the methods.

3.3.2. Basic Principle

When electron/X-ray beams are generated and then impact on the sample, the incident beams will collide with electrons of the atoms of the sample and produce various quanta, including Auger electrons, secondary electrons, elastically/inelastically scattered electrons, and X-rays (Bremsstrahlung, characteristic X-Rays, and fluorescent X-rays). Figure 3–3 illustrates the general principle of the emission of various quanta generated from the excitation area of the surface. The beam penetration into the sample surface is determined by four major parameters: the acceleration voltage, the beam current, the beam spot size, and the atom number (orbitals) of the sample. The electrons, X-rays and/or photons emitted from the surface can be collected, detected and evaluated in several ways, whereby various characterization methods can be applied to the surface region of the titanium implants.

3.3.3. Surface Structure - Scanning Electron Microscope (SEM)

Surface structure, morphology, and topography can be broadly defined as the configuration of a surface. In practice, however, the terms are often used together to describe the surface texture. Scanning electron microscopy (SEM) is the most widely used technique to characterize the surface structure (morphology and qualitative topography) of screw-shaped dental implants. SEM images can be obtained by detecting the secondary and backscattering electrons ejected from the surface region that the accelerated primary electrons probe (see below). Another type of SEM, stereo-scanning electron microscopy (stereo-SEM), enables us to measure the quantitative topography, such as the roughness values. In principle, the stereo-SEM uses stereoscopic pairs of micrographs obtained by tilting the sample or the electron beam in an SEM chamber.

Table 3–1. Surface characterization of screw-shaped titanium implants

Surface characteristics		Machine-turned implant	TiOblast	Osseotite	SLA
Modification method		CNC machine-turned	blasting	double acid etching	blasting and acid etching
Chemical composition		Mainly TiO ₂ , and traces: C, Na, Si	Mainly TiO ₂ . Contaminat C ≤ 28 at%, Traces: N	Mainly TiO ₂ . Contaminat C ≤ 32 at%, Traces: N	Mainly TiO ₂ . Contaminat C ≤ 23 at%, Traces: N
Morphology		Machine-turning oriented grooves ≤ 10 μm and a wave length ≈ 30-50 μm. Occasional pits and protrusions	No grain pattern (x1,000) Anisotropic grit-blast pits with facets (x 10,000)	Stronger grain pattern than others (× 1,000) Anisotropic etch pits with needle-like boundaries at high magnification (× 10,000)	Distinct grain-like pattern (x 1,000) Anisotropic etch pits but smooth boundaries compared to the osseotite surface at high magnification (× 10,000)
Pore/pit size		NA	≤ 2 μm	≤ 2 μm	≤ 2 μm
Oxide thickness	porous film	Air-formed thin oxide film	Air-formed thin oxide film	Air-formed thin oxide film	Air-formed thin oxide film
	barrier film	17 ± 6 nm at thread peak, flange and valley			
Crystal structure		Amorphous	Amorphous	Amorphous	Amorphous
Roughness	Sa, μm	0.68 (±0.32)	0.92 (± 0.34)	0.72 (± 0.43)	1.28 (± 0.25)
	Sdr, %	1.23 (± 0.01)	1.29 (± 0.10)	1.29 (± 0.16)	1.32 (± 0.06)
	Sds, μm ⁻²	0.09 (± 0.03)	0.08 (± 0.04)	0.12 (± 0.05)	0.07 (± 0.01)

Surface characteristics		TiUnite	Ca implants	Mg implant	Nanotube implants
Modification method		MAO and anions incorporation	MAO and cations incorporation	MAO and cations incorporation	Potentiostatic electrochemical oxidation
Chemical composition		Mainly TiO ₂ , P ≤ 8 at%. Contaminant: C ≤ 26 at%, Traces: N	Mainly TiO ₂ , Ca ≤ 8 at%. Contaminant: C, Traces: N and F	Mainly TiO ₂ , Mg ≤ 8 at%, Contaminant: C ≤ 19 at%. Traces: N, Na	Fluorinated titanium oxide nanotubes of F-TiO ₂ , TiOF ₂ , and F-Ti-O with F ≈ 5 at.% Contaminants C ≤ 27, P ≤ 1.3 at.%. Traces: N, Ca
Morphology		Duplex oxide structure The outer porous film with micropores and the inner barrier film without micropores	Duplex oxide structure The outer porous film with micropores and the inner barrier film without micropores	Duplex oxide structure The outer porous film with micropores and the inner barrier film without micropores	Nanotubular structure (x100,000) Nanotubes of ≈ 700 nm in length. Porosity ≈ 60%.
Pore/pit size		≤ 4 μm, elongated	≤ 1.5 μm, elongated	≤ 2 μm	Pore size distribution ≈ 58 - 150 nm
Oxide thickness	porous film	<u>Heterogeneous</u> 5.7 μm at the 1st thread 5.9 μm at the 3rd thread 9.3 μm at the 5th thread	Homogeneous ≈ 4 μm (± 0.6) at the all threads	Homogeneous 3.4 μm (± 0.1) at the all threads	Homogeneous 700 nm
	barrier film	0.9 - 5.0 μm	1.5 - 2.0 μm	1.3 - 2 μm	
Crystal structure		Anatase + rutile	Anatase + rutile	Anatase + rutile	Amorphous
Roughness	Sa, μm	1.34 (± 0.16)	0.64 (± 0.16)	0.69 (± 0.24)	0.65 (±0.02)
	Sdr, %	1.25 (± 0.37)	1.28 (± 0.11)	1.26 (± 0.11)	1.43 (±0.9)
	Sds, μm ⁻²	0.06 (± 0.01)	0.09 (± 0.03)	0.12 (± 0.04)	0.06 (± 0.04)

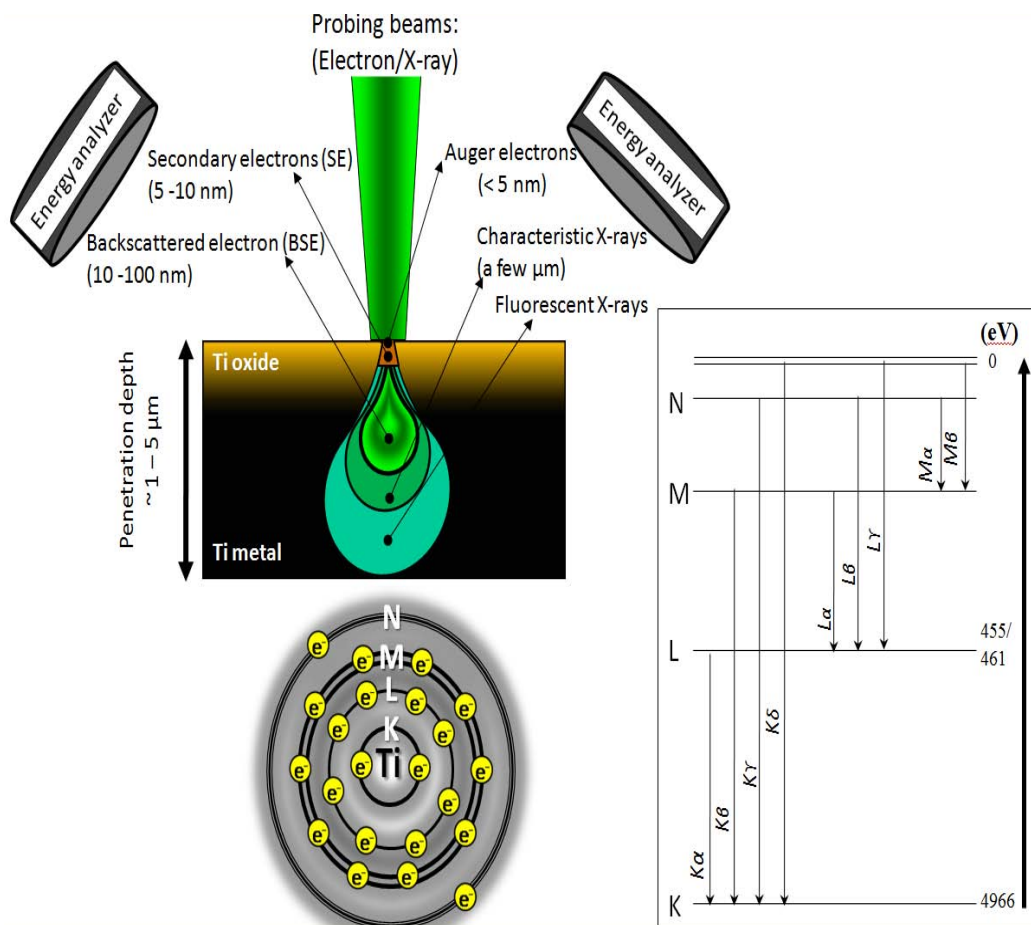


Figure 3-3. Illustrations of emission and spatial resolutions of various quanta. The escape depth of electrons from the sample surfaces is the following energy order: Auger electrons < secondary electrons < backscattered electrons < characteristic X-rays. (For details, see text).

For the measurement of quantitative topography of screw-shaped dental implants, stereo-SEM techniques can obtain the benefits of superior high spatial resolution of a few nanometers to a few hundred of nanometers compared to the other techniques, which use light sources; the difference arises from the electron diffraction vs. the light reflection in interactions with the surface region. Technical difficulties of either exactly superimposing stereoscopic pairs or tilting the beam or sample are encountered. Furthermore, advances in conventional SEM provide field emission scanning electron microscopy (FE-SEM) with ultra-high resolution and also provide focused ion beam scanning electron microscopy (FIB-SEM) for rapid cross-section (window openings) of a small target area. In general, the physical principle remains the same when taking images (see below).

Figure 3-4 shows SEM images, for both the planar and cross-sectional views, of the micro- and nano-structured surfaces produced by various surface engineering methods, involving in Tioblast[®], Osseotite[®], SLA[®], Tiunite[®], Ospol[®] and Implant M[®], Osseospeed[®], nanotite[®], and nanotubes implants.

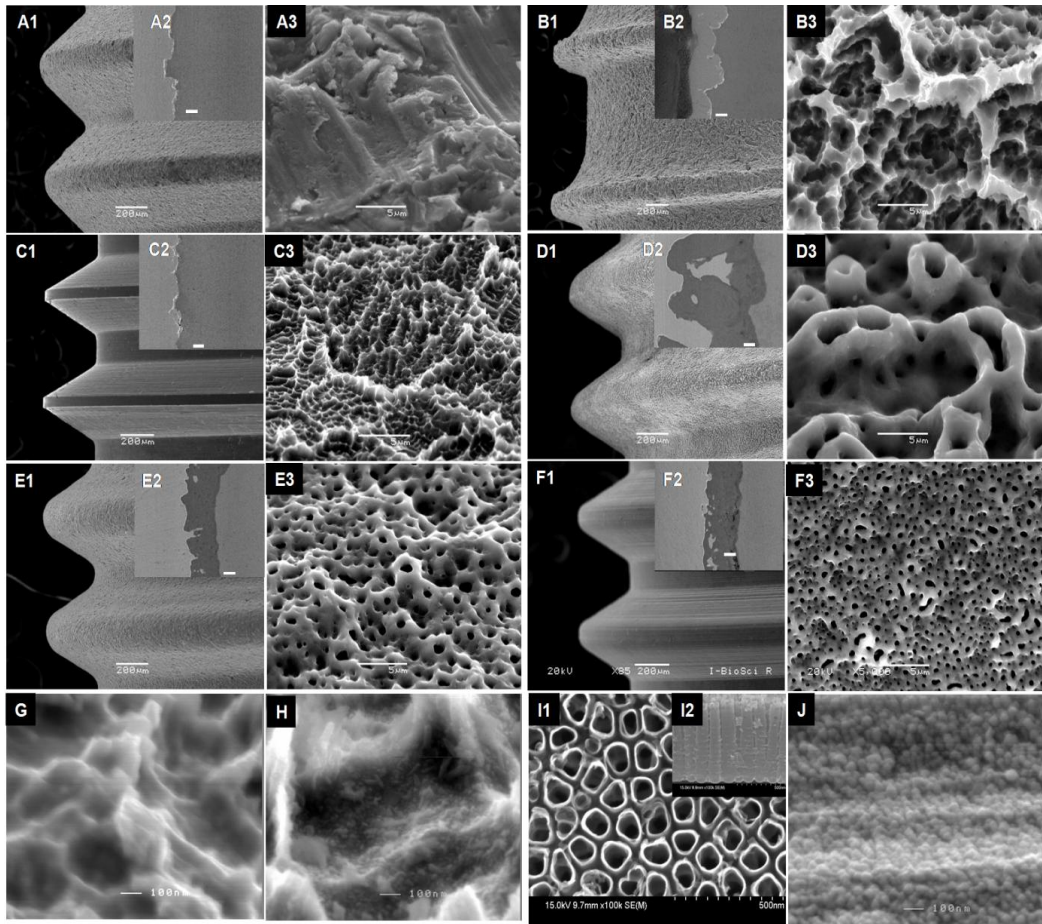


Figure 3–4. SEM and FESEM images show a variety of micro- and nano-structured surface topographies that are primarily dependent on the surface engineering techniques in use. The images were taken at different tilting angles, from 15° to 30° , and at different acceleration voltages from 3 KeV to 20 KeV, optimizing the surface properties of the micro- and nanomaterials. The scale bars are 1 μm for the inset images in A2 – F2, 200 μm for the low magnification images in A1 - F1, 5 μm for the high magnification images in A3 - F3, and 100 nm for FE SEM images of the nanomaterials in G – J. The oxide thicknesses are measured on cross-sectional view (inset). A) blasted Tioblast[®], B) blasted and acid etched SLA[®], C) dual acid etched Osseotite[®], D) oxidized, phosphorous-incorporated TiUnite[®], E) oxidized, calcium-incorporated Ospol[®], F) oxidized, magnesium-incorporated Implant M[®], G) blasted and acid etched Osseospeed[®], H) calcium phosphate/hydroxyapatite precipitated Nanotite[®], I) blasted, highly ordered TiO₂ nanotubes Implants [23,24], and J) nano hydroxyapatite assembled implants. The surface chemistry of these implants is shown in Figures 3–16, 3–17, 3–18 and Table 3–3.

3.3.3.1. Scanning Electron Microscope (SEM)

Figure 3–5 presents a schematic diagram conventionally observed in most SEMs. When the incident electron beam interacts with the sample mounted, there will be two types of collisions. One type is the elastic collision, and the other type is the inelastic collision. Elastic scattering occurs between the negative electron and the positive nucleus, resulting in little ($<1\text{eV}$) or no change in energy of the scattered electron. The typical angle of scattering is approximately five degrees of change in momentum. The main processes during inelastic scattering include phonon and plasmon excitation, the generation of X-rays and secondary electrons, and ionization of inner shells. The SEM technique provides sample information of

the image (topography, morphology), the elemental composition, and the electronic properties by detecting and analyzing the electron and/or X-rays and photons. There are several types of SEMs, depending on the signal generated, such as the environmental SEM (ESEM), FE-SEM, and stereo-SEM. The common signals in use in most SEMs are the secondary electrons (SE), back-scattered electrons (BSE), characteristic X-rays, and light (cathodoluminescence). The column and scope chamber of an SEM contains the following components:

Electron gun: Electron source at the top, producing a beam of monochromatic electrons.

Anode plate: Engages high-voltage differential from the cathode, accelerating the electron beam down the column.

The first condenser lens system: Condenses the electron beam, usually by the "coarse probe current knob," and produces a reduced spot size.

The second condenser lens system: Further condenses the electron beams as thin, tight, coherent spots, possible usually by control of the "fine probe current knob."

Scan coils are a set of coils that electromagnetically sweep the electron beam in a grid fashion on the sample.

Final objective lens system: This system focuses the scanning beam on the surface of the sample desired. The smallest spot is approximately 2-5 nanometers.

Detectors - In the scope chamber

Secondary electron detector – Used for taking the secondary electron image

Backscattered electron detector – Used for taking the backscattered electron image

Energy dispersive detector – Used in EDS

Sample mount and stage: In the scope chamber, move the sample to the position desired.

The secondary electrons are sample electrons that are obtained by inelastic collisions of the probed electron beam with weakly bonded conduction-band or valence electrons of the surface atoms in the sample. An SEM image is primarily obtained by a secondary electron detector and displayed on a computer monitor, as shown in Figure 3–5. Magnification of an SEM image ranges from approximately 10 to 500,000 times and can be controlled by the key parameters of current or voltage. To obtain the secondary electrons, the incident electron beam must be first generated by the electron gun. The electron gun consists of three parts: the filament (alternatively the cathode or emitter), the shield, and the anode. The overall function of the electron gun is to provide an intense beam of high-energy electrons to generate as small a "spot" as possible. The shield contains a Wehnelt cylinder, a Wehnelt cap, and a gun cap. The electron resource is crucial for determining the SEM quality. There are two major types of electron emitters; one type is a thermionic electron emitter such as tungsten filaments and crystals of Lanthanum hexaboride (LaB₆), and the other type is a field emitter (field emission gun used in FESEMs). The operation of a thermionic electron emitter is based on the principle that as a filament heats up, it emits electrons in the outer orbitals of the component atoms of the filament materials. Tungsten filaments have been widely used because they yield a large number of electrons as they are heated, and they have the highest melting point of all of the metals. Tungsten filaments give relatively low brightness, probe size ~ 4.0-8.0 nm, Φ (work function) = 4.5 eV, life time ~ 70 hours. A LaB₆ emitter is smaller in size and geometry than the tungsten filaments, which leads to an increase in the signal-to-noise ratio (S/N), Φ = 2.7 eV, life time ~ 350 hours, and higher brightness. Unlike a thermionic electron emitter in which the electron source is heated up, the field emission gun in an FESEM works

instead on the principle that electrons are pulled down by a potential field that is generated by the anode beneath the filament tip, resulting in a much smaller primary excitation zone. Hence, use of the field emitter in FESEM provides great benefits over a conventional SEM using thermionic emitters, having a smaller initial spot size (< 3.0 nm vs. 4.0-8.0 nm), a lower accelerating voltage (2-5 KeV vs. 5 - 30 KeV), and a much higher resolution (< 500.000 vs. < 100.000).

The lens system in SEMs aims to condense and optimize the electron beam as a thin, tight, focused spot on the surface of the sample. The condenser lens serves to condense the primary electron beam with an energy ranging from 0.5 keV to 40 keV, which is in turn focused to a spot approximately 4 Å to 5 nm in diameter. The beam passes through the paired scan coils in the electron column. The scan coils are essentially electromagnetic materials and are set in two pairs. One pair controls the beam moving around the X axis, and the other pair deflects the beam in the Y axis. Thus, the electron beam scans across a rectangular area over the sample surface (raster scanning). The lenses and electron optical systems inherently produce several different types of aberration, including chromatic aberration, spherical aberration, aperture diffraction, and astigmatism. These aberrations can affect the image quality obtained in the SEMs. Those aberrations that are inherent to the electron beam are electronically corrected by controlling a set of electromagnets provided in different ways from the manufacturers.

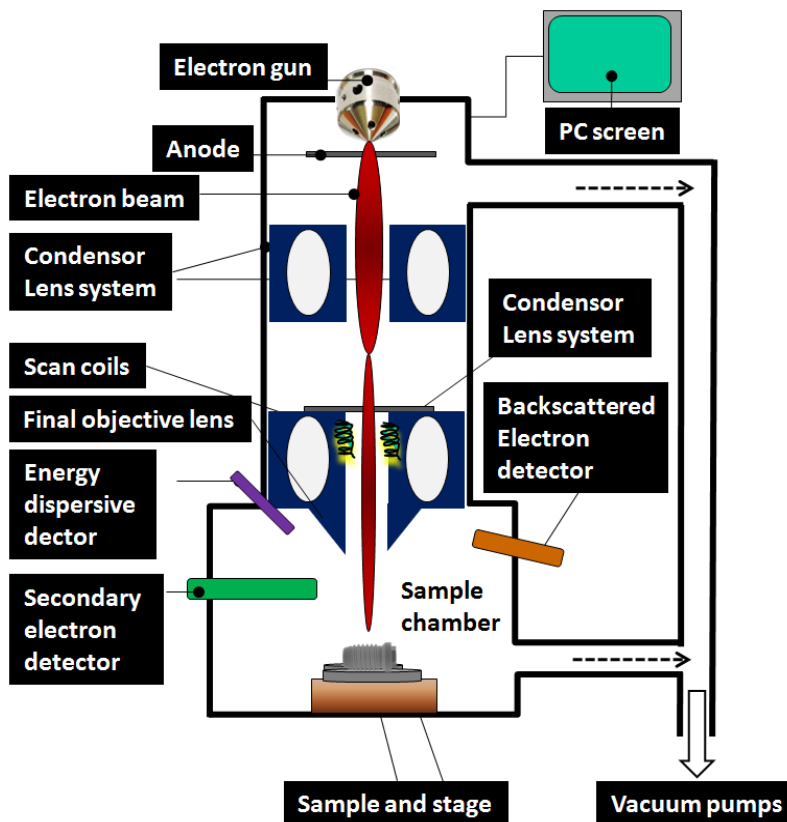


Figure 3–5. Schematic diagram of a scanning electron microscope. (For details, see text).

The primary electron beam focused through the lens systems in the column impacts on the sample. As shown in Figure 3–3, the interaction between the incident electron beam and the sample generates a variety of signals that are utilized for various characterization methods, depending on the acquisition of the signals. The accelerated primary electron beam in the very beginning of contact with a sample surface is not immediately bounced off but rather penetrates into the sample for some distance before it collides with the orbital electrons of the sample atoms and creates a “tear-drop” shaped primary excitation zone. D. C. Joy, in 1991, published hypothetical electron trajectories in bulk samples by using “Monte Carlo–based electron–solid interaction simulations” [24]. Figure 3–6 shows Monte Carlo simulation of electron trajectories in bulk samples of carbon, aluminum, and gold as a function of the primary electron beam energy at 5 keV, 15 keV, and 20 keV. The importance of understanding the incident electron beam/sample interaction is underscored by the significant changes of the spatial and depth distribution (hence determining depth and spatial resolution) shown in Figure 3–5.

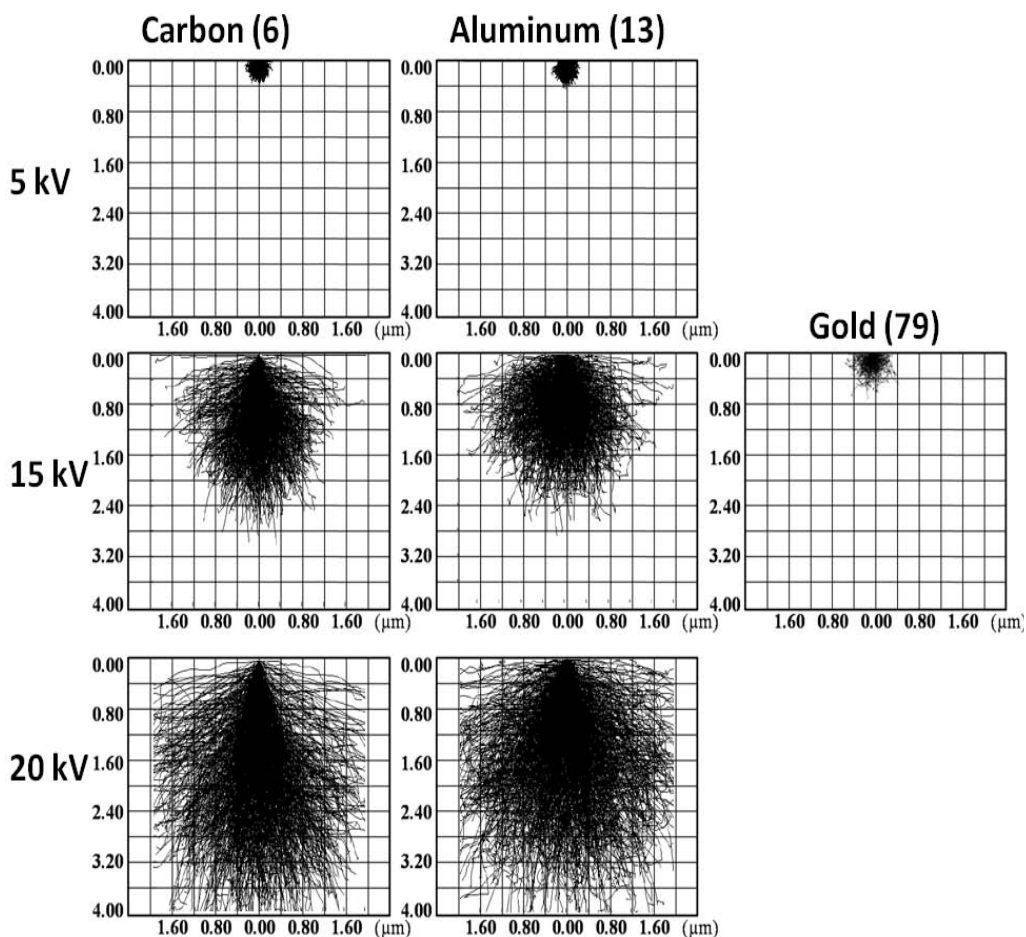


Figure 3–6. Monte Carlo simulation of electron trajectories in bulk samples of carbon, aluminum, and gold at 5 keV, 15 keV, and 20 keV.

As a consequence, the primary electron beam is very narrow. The very narrow electron beam provides a large depth of field and enables the secondary electron to produce ultra-high-resolution images of a sample surface, less than 5 nm in a spatial resolution. Thus, the secondary electron is the most widely utilized signal in SEMs for characterization of the surface topography, morphology and structure of the dental implant. Secondary electrons at a given instrumental condition have a low electron energy $E_k \leq 50$ eV that is typically ejected from the k-orbitals of the surface atoms or the near-surface of the sample by inelastic scattering interactions with beam electrons of the penetration depth ≤ 10 nm. In general, the secondary electrons are more readily produced in the case of elements with a higher atomic number than in the case of elements with a low atomic number. The number of secondary electrons reaching the detector is strongly related to the size of the primary electron beam, the acceleration voltage, the beam current, the vacuum condition, the composition of the sample, and the condition of sample preparation, which determine the topographical image quality of a sample. For instance, increasing the size of the beam yields a better signal to noise (S/N ratio), but the larger beam diameter results in a lower resolution. The escape path of the electron beam decreases with an increase in the incident angle, and therefore, secondary electrons of the electron beam will be increasingly emitted. For this reason, a surface represents a brighter area at the margins/edges and a darker area at the bottom/valley of its irregularity. When probing with an electron beam on the screw-shaped dental implants, the plane of the flange, peak, and valley of the implant threads needs to be tilted for the beam to be vertically focused on them, as illustrated in Figure 3–7.

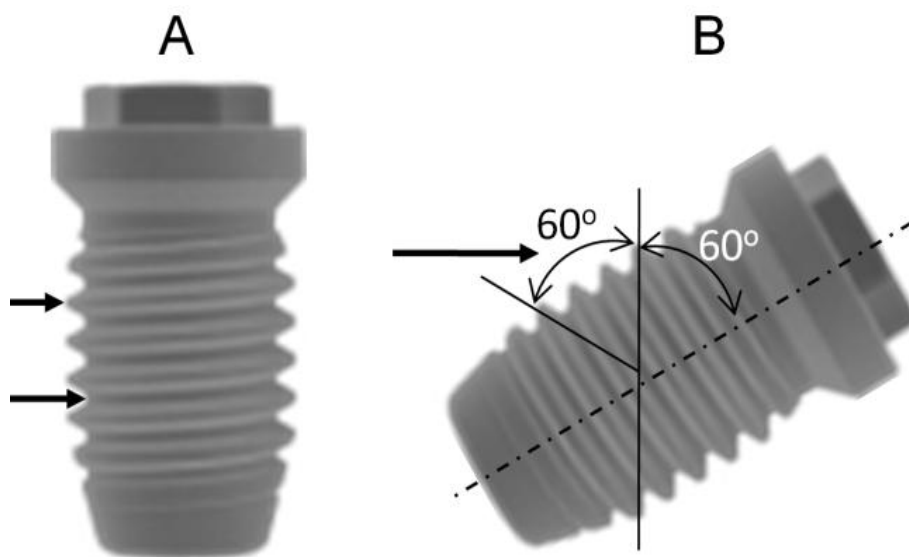


Figure 3–7. Evaluation of the surface characteristics of screw-shaped dental implants is sometimes limited due to their screw geometries. To vertically focus to the plane of the flange, peak and valley of the implant threads, the screw-shaped implants need to be tilted in many cases of surface characterization techniques. A) a primary electron beam is focused on the peak and valley. B) an electron beam is vertically focused on the plane of a thread flange by tilting the implant sample as much as the thread angle. The example shows 60° tilting of the implants.

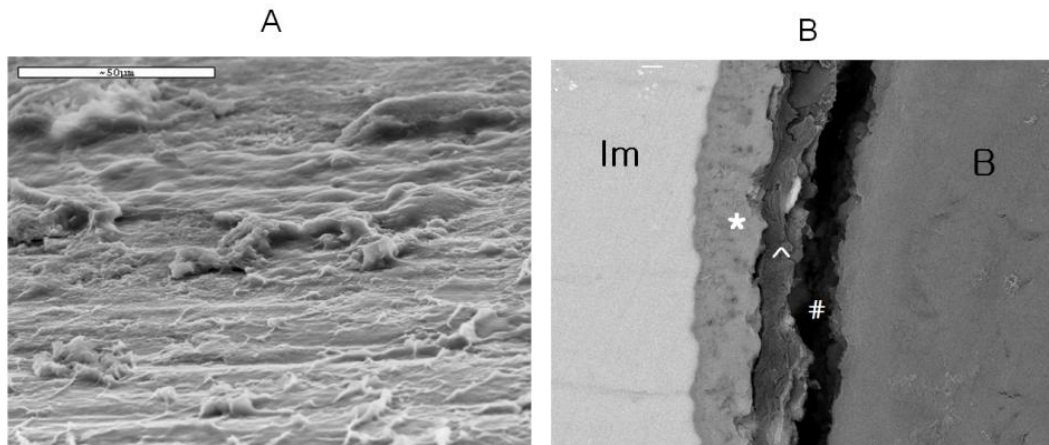


Figure 3–8. Backscattering images [6] of A) the retrieved implant from rabbit femur by unscrewing with removal; torque measurement shows the bone/implant interface with a tilting angle of 30° , and B) the cross-section view of the bone/implant surface interface allows us to distinguish the titanium material (Im), titanium oxide (*), immature woven bone (^), fracture line between the woven bone and mature bone, bone matrix (B), and bone cells from the background resin material, because the intensity of the BSE signal differs in accordance to the atomic number of the components.

The back-scattered electrons (BSEs) are primary beam electrons that are reflected or back-scattered out of the near surface ≤ 100 nm (approximately) of a sample by elastic scattering but will also undergo several inelastic collisions. BSEs have a greater energy than SEs and therefore are capable of escaping from greater depths and diameters of the “tear drop” near the sample surface than ≤ 10 nm (approximately) of the SEs, as shown in Figure 3–3. The yield of the backscattered electron tends to increase monotonically with the atomic number (Z) of the sample. Therefore, BSE imaging can provide information about chemical composition resulting from the differences of the atom number of the sample. In addition, the yield of the secondary electron depends on the beam energy, the incident angle, the surface irregularity, and the crystallinity. A good example in implant research for applying the principle of dependence of backscattering electrons on the atom number is in high resolution investigations of the interface between the bone and implant surface. As shown in Figure 3–8, a backscattering image of the resin-embedded bone/implant interface in the cross-sectional view shows distinctions between the titanium material, titanium oxide, immature woven bone, bone matrix, and bone cells from the background resin material. [6]

A vacuum environment is very necessary in SEMs system. The pumping system can be facilitated in the range of 10^{-5} to 10^{-9} Torr in low, high-*vacuum* Ultra-High Vacuum (UHV) modes. A major reason for requiring a vacuum is because we can effectively utilize the intense beam of high-energy electrons striking on the sample in the system and because the vacuum will ultimately improve the quality of the image.

The sample must be of a size that fits on the sample mount. The dental implants in use commercially can be appropriately mounted in most SEMs. To prevent the accumulation of electrostatic charge at the surface, i.e., charging, which causes scanning faults and results in poor images, samples must be electrically conductive. In addition, coating can dissipate a heat generated on the surface by electron bombardment and also serves to maintain the stability of soft biological samples such as cells. Nonconductive samples are therefore usually coated by vacuum evaporation of a sputter coater with electrically conducting materials, commonly

gold, platinum, and carbon. Coating increases the signal intensity and therefore improves the surface resolution. However, it should be noted that the coating of materials may result in differences in signal generation. The coating of heavy metal with a high atom number such as gold or platinum can more easily produce secondary electrons compared to coating of the carbon with a low atomic number but tends to disturb the generation of the backscattered electrons or the characteristic X-rays. Therefore, coating materials should be selected in accordance with which signal is of primary interest for the surface characterization. For example, if surface structure analysis is the main purpose, the heavy metals can be used as coating materials, while if characterization of the chemical composition is of primary interest, then carbon is preferred as a coating material.

When a setting for the gun current, the acceleration voltage, and the spot size is given, with a correction for astigmatism and adjustment of the working distance and focus, then contrast and brightness can be manually controlled and optimized to obtain high-quality images and data, depending on the skill level and experience of the instrument user.

As shown in Figure 3–4, blasted, etched, and blasted/etched implants including Tioblast[®], Osseotite[®], and SLA[®] showed thin oxide layers of approximately 3 - 5 nm, with micro-pits varying in size by approximately 1 μm . Electrochemically oxidized implants such as TiUnite[®], Ospan[®] and Implant M[®] showed the microporous structures of duplex surface oxides with pore size $\approx$ 1 - 10 μm in at least one direction. The thick oxide layer was in the range of 3 - 10 μm , and the crystal structures are characterized phase transformations known as anatase, rutile or mixtures. Importantly, in addition to changes in the surface structure, the surface chemistry changes should be noted in the cases of TiUnite[®], Ospan[®] and Implant M[®]. Nanotite[®] is an example of emerging surface nanotechnology in titanium implant dentistry. They present nanostructured surfaces with nanopits $\leq$ 150 nm and irregular shapes of nanoparticles $\leq$ 100 nm, respectively. There may be misleading perceptions due to poor surface characterizations mentioned above. For instance, when one measures quantitative and qualitative nanotopography of the nanomaterials that are smaller than 100 nm in at least one dimension, the use of equipment that has a spatial resolution of, at best, $\geq$ 130 nm will yield inadequate measurement values, which in turn give rise to certain misconceptions about *in vitro* and *in vivo* effects of the nanomaterials. The surface chemistry is characterized as mainly TiO₂ in Osseospeed[®] and hydroxyapatite/calcium phosphate in Nanotite[®]. Regarding surface chemistry changes of those implants, more details will be described in the section on surface chemistry below. For more information, the interested reader can find useful references [25-31] and website resources. [32,33]

3.3.4. Chemical Composition – EDS, AES, and XPS

The surface chemistry of dental implants is a parameter of great importance for the improvement of osseointegration. To understand chemical properties of the implant surface, EDS, AES, and XPS have often been used in basic research on implant dentistry. The methods use different principles to characterize the chemical properties. Figure 3–9 illustrates the basic principles that generate characteristic X-rays in an EDS system, Auger electrons in AES systems, and photoelectrons in XPS systems from the surface atoms.

3.3.4.1. Energy-dispersive X-ray Spectroscopy (EDS or EDX)

EDS analysis is non-destructive and provides a qualitative identification of the elements and semi-quantitative information on relative concentrations of the elements. An EDS analysis system is very often equipped with SEMs, named SEM-EDS, which are used for compositional analysis of the “near” surface of the sample, but is not of the actual surface *per se*, because of the electron path (penetration and escape) in use (Figures 3–3 and 3–6). Characteristic X-rays have greater energy than either Auger electrons, secondary electrons, or backscattered electrons and therefore are able to escape from greater depths and diameters of the “tear drop” in the sample surface than the ≤ 5 nm (approximately) of the Auger electrons, ≤ 10 nm of the secondary electrons, and ≤ 100 nm of the backscattered electrons. In this context, EDS is certainly not a surface analysis technique.

A principle of EDS elemental analysis is based on Moseley’s Law, implying that the higher the atomic number of an atom is, the higher the energy of the characteristic X-Rays emitted from a given electron shell line will be. When the primary electrons collide with the surface atoms, thus ejecting an electron of a low-energy inner shell of the atoms, this process leaves a hole or a vacancy in the shell; consequently, a high-energy electron in the outer shell jumps down into the inner shell and fills the hole or vacancy, making a shell transition. The energy difference (ΔE) between the inner and outer orbitals by shell transitions such as K, L, M, and N (Figure 3–3) will be transferred to X-ray emission, e.g., characteristic X-Rays, continuum X-Rays (Bremsstrahlung), and secondary fluorescence X-Rays. An EDS analysis system utilizes the characteristic X-Rays for chemical analysis of the implant surface.

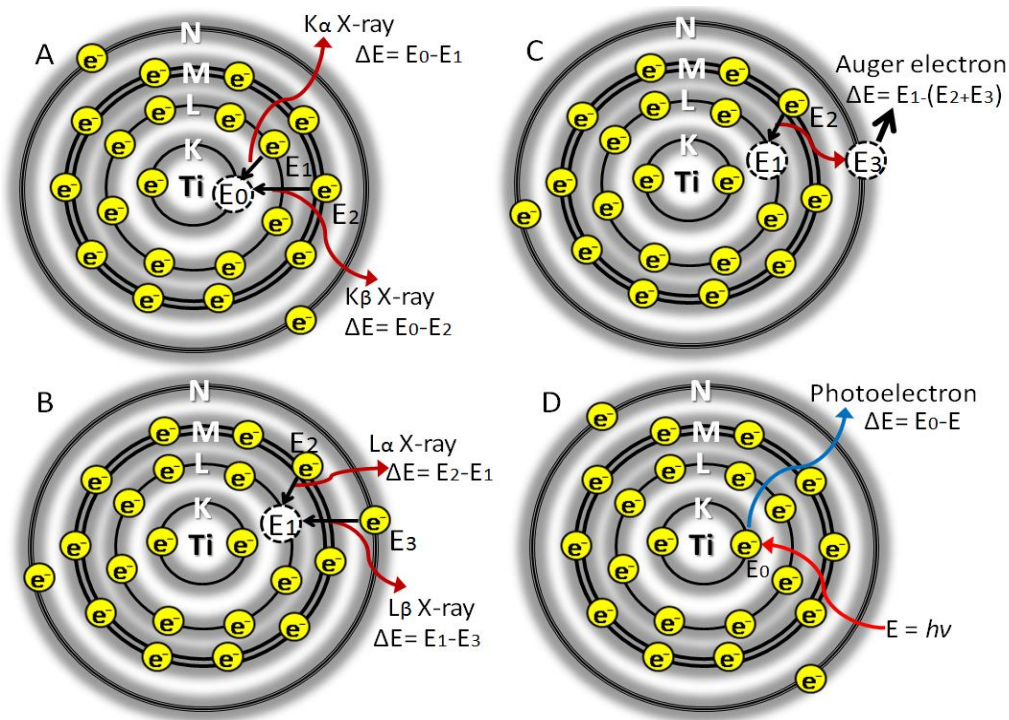


Figure 3–9. The cartoon illustrates electron transitions of the titanium atom that generates characteristic X-rays, A) Kα, β family and B) Lα, β family that are utilized in an EDS system, C) Auger electron that is applied to AES, and D) photoelectron that is used for XPS analysis. (For details, see text).

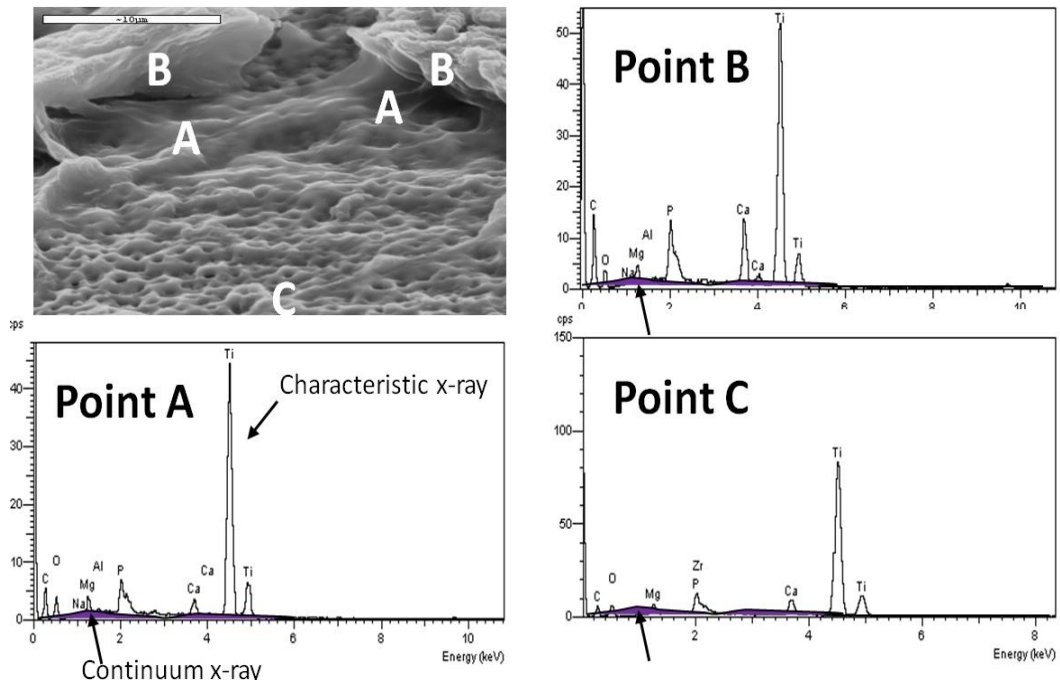


Figure 3–10. EDS point analysis of chemical elements at A, B, and C regions of the retrieved implants from rabbit femur after six weeks of follow-up. Continuum X-ray (marked by arrows) is detected near baselines in the EDS spectra. EDS point analysis measures an exact area with a very narrow point over the AES or XPS measurements. (For details, see text).

Let's take the K (α , β , γ) shell transition. The electron vacancy in the K shell of the sample surface generated by an incident primary electron with a high energy (E_0) is usually filled by an electron from an outer shell, such as L, M, or N (Figure 3–9), resulting in a characteristic X-ray quantum. This series of transitions is called an electron cascade: A K_α X-Ray will be emitted when the K shell vacancy is filled by an L shell electron, a K_β X-Ray will be emitted when the K shell vacancy is filled by an M shell electron, and a K_γ X-Ray will be emitted when the K shell vacancy is filled by an N shell electron. The energy (E_x) of the characteristic X-rays is expressed as the following:

$$E_x = E_K - E_{L,M,N}$$

where E_K is an electron energy of the K shell, and $E_{L,M,N}$ is an electron energy of the L, M, or N shell. The K_α characteristic X-rays are most often used in EDS analysis because of their inherent high-energy intensity. The detector in an EDS system can absorb low-energy X-rays. Therefore, the atoms that have an atomic number less than 4, i.e., H, He, and Li, cannot be detected. [27]. In this way, an EDS analysis gives us chemical information on the sample by interpretation of the characteristic X-ray peaks according to a specific atom (atomic number). Figure 3–10 illustrates characteristic X-ray spectra of the K series peaks according to the atomic number of the atoms that compose the implant surface retrieved from animal bone after six weeks of follow-up. The electron beam can be precisely controlled, thus enabling us point analysis of the EDS spectra on the desired regions such as a specific point, particles or

defects on the sample surface. The examples of point analysis on a retrieved implant's surface in Figure 3–10 show the distribution of the chemical elements at an exact area. In addition, the EDS technique provides line profile analysis of the elemental distribution across the surface.

Figure 3–11 shows an example of line profile analysis at the interface between the bone and the retrieved implants. Line profile analysis in EDS was used to investigate whether ionic movements/gradients may occur at the bone/implant surface interface when an implant is inserted into the bone. Figure 3–12 shows changes of the peak intensities of EDS spectra at Mg K α , P K α , Ca K α , Ti K α , N K α , S K α , O K α , and C K α . The study indicated that the technique of using line and point analysis in an EDS system benefits greatly from obtaining chemical information at a narrow area such as a bone/implant interface because the electron beam can be focused in a very small spot as compared to the other X-ray resource methods such as AES and XPS, which have a relatively wide spatial resolution area (See the section of AES and XPS) [6].

3.3.4.2. Auger Electron Spectroscopy (AES)

AES analysis is a surface sensitive technique to be used for quantitative and qualitative analysis of the surface composition on the basis of measuring the kinetic energies of Auger electrons, named after Pierre Victor Auger, a French Physicist, who first observed the effect in the mid-1920s. Auger electrons are emitted as a consequence of shell transitions following the ejection of a core electron (Figure 3–9c). As schematically illustrated in Figure 3–13, the Auger emission process is initiated from the creation of an electron vacancy in a low-energy inner shell of the atoms (here: L_{2, 3}) by an incident primary electron with a high energy sufficient to ionize core shells (Ionization: Step 1). Subsequently, the vacancy is filled by a high-energy electron dropping from an outer shell (here: M_{2, 3} or M_{4, 5}) (Relaxation: Step 2). The energy difference in this process is transferred to the emission of an Auger electron (Auger emission: Step 3). The kinetic energy of an Auger electron (E_{Auger}) is formulated as follows:

$$E_{\text{LMM Auger}} = E_{\text{L3}} - (E_{\text{M2,3}} + E_{\text{M4,5}})$$

where E_{L3} is the energy of an electron in L₃, $E_{\text{M2,3}}$ is the energy of an electron in M_{2,3}, and $E_{\text{M4,5}}$ is the energy of an electron in M_{4,5}. However, it cannot be distinguished which electrons (M_{2,3} or M_{4,5}) fill the electron vacancy of the inner shell (e.g., the L shell). Therefore, an Auger transition is primarily determined by the electron locations of the inner and outer shells of the sample atom. Core electron vacancies (ionization) are initiated by using a high energy of a primary electron beam typically in the range of 2 - 10 keV or soft X-rays in the range of 1 -2 eV, called XAES, and creating Auger electrons that have lower energy than the incident electron beams or X-rays. A probing beam generates Auger electrons in three pathways: i) Auger electron emission by the incident beam per se, ii) Auger electron emission by backscattered electrons at the very surface, and iii) Auger electrons are excited, but are not able to be emitted due to the depth. Hence, Auger electrons undergo a very narrow escape path near the very surface of the sample, typically at an escape depth $\approx$ 0.1 - 5 nm. Auger electrons are emitted from a shallower depth and a narrower diameter, as shown in the “tear drop” (Figure 3–3), compared to the secondary electrons and backscattered electrons.

Such a shallow and narrow electron path empowers the AES to be a very useful technique for determining the chemistry of the top few atomic layers or the first molecular layer of the uppermost surface of the sample. By using a broad-focused electron beam probe, however, compositional data can be obtained from a relatively large surface area ($\sim 1\text{mm}^2$) of interest.

In general, the electron shells are labeled K, L, M, N, O, P, and Q, which is designated $n = 1, 2, 3, 4, 5, 6$, and 7 , respectively (Table 3–2). Auger transitions are expressed in a combination of three capital letters above mentioned, i.e., KLL, KLM, LMM, LVV (V = valence bands). The KLL shell may have six Auger transition notations, i.e., KL_1L_1 , KL_1L_2 , KL_1L_3 , KL_2L_2 , KL_2L_3 , and KL_3L_3 . We will take an example and clarify the Auger transition notation mentioned above, $\text{L}_{2,3}\text{M}_{2,3}\text{M}_{4,5}$. The first letter, $\text{L}_{2,3}$, refers to the electron shell in which an initial core vacancy occurred. The second letter, $\text{M}_{2,3}$, indicates the shell that an electron comes from to fill the initial electron vacancy. The third letter, $\text{M}_{4,5}$, refers to the shell that the Auger electron is emitted from. Thus, compositional atoms of the sample surface will give rise to characteristic peaks of the spectrum at their various kinetic energies. Titanium is a $3d$ transition metal, which characterizes the “fingerprint” of the LMM triplet, consisting of the transitions L_3VV , $\text{L}_3\text{M}_{2,3}\text{V}$, $\text{L}_3\text{M}_{2,3}\text{M}_{2,3}$ in the order of decreasing energy. Figure 3–14a shows typical AES survey spectra obtained from machine-turned implants and anodized titanium implants, consisting of the peaks of LMM at $\approx 389\text{ eV}$ and LMV at $\approx 422\text{ eV}$ of the titanium atom, a peak of KLL at $\approx 517\text{ eV}$ of the oxygen atom. Auger transitions can take place in all atoms except for H (hydrogen) and He (helium), but are most sensitive to the low atomic number elements, typically ca. 1% monolayer, while the XPS process is more sensitive to high atomic number elements. For this reason, the Auger method is favored to use for low atomic number elements.

Auger spectra are often plotted as the differential of the electron signal, $dN(E)/dE$, or $d[E \times N(E)]/dE$, where the total Auger electron signal, $N(E)$, electron energy, E . AES must be performed in UHV (ultra-high vacuum) conditions, generally accepted as approximately 10^{-8} or 10^{-9} torr. The low pressure enables us to obtain more accurate data. AES data interpretation is carried out in comparison to well-known fingerprint peaks of Auger transition energy for the standard atoms. A number of online databases provide reference information about spectra, line positions, chemical bond energies and sensitivity factors for a wide variety of elements and compounds related to AES and XPS analysis [26–50]. However, Auger transition energy inherently involves three electron shells such as KL_1L_1 , KL_1L_2 , KL_1L_3 , KL_2L_2 , KL_2L_3 , KL_3L_3 , or $\text{L}_{2,3}\text{M}_{2,3}\text{M}_{4,5}$, which leads to more complicated patterns due to chemical shifts, thus making AES data interpretation more difficult than XPS data.

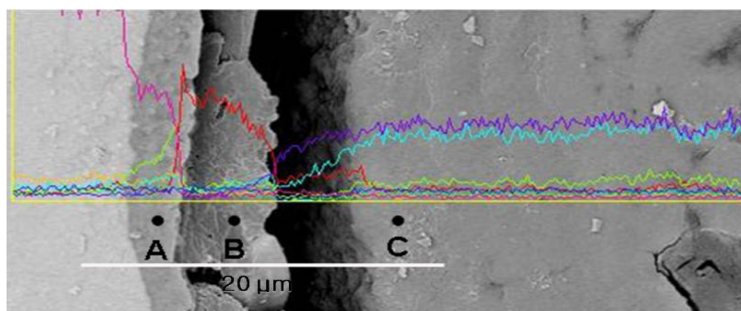


Figure 3–11. EDS line profile shows the elemental distribution along the line measured on the cross-section of the retrieved implant from the rabbit bone after six weeks of follow-up.

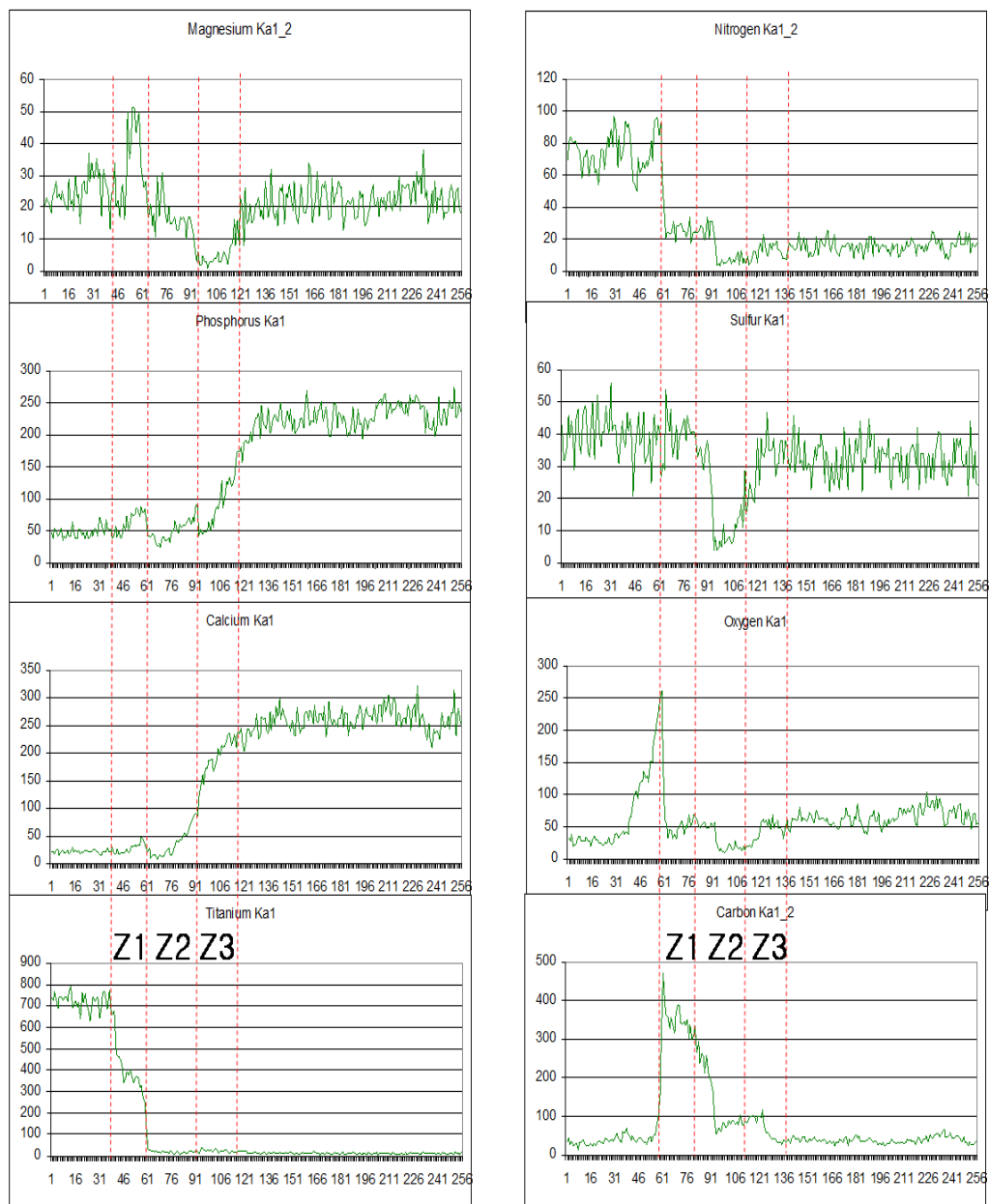


Figure 3–12. The spectra of the EDS line profile in Figure 3–11 shows changes of the peak intensities of Mg K α , P K α , Ca K α , Ti K α , N K α , S K α , O K α , and C K α at the oxidized, bioactive implant surface/bone interface in preparation for cross-sectioning, which indicates ionic movements/exchanges at the interface, providing evidence to support biochemical bonding theory[6].

Because the sample surface is readily contaminated with carbon and oxygen from air exposure, there is a need to clean up the contaminants by sputtering (or etching), i.e., sputter cleaning, where a beam of Ar⁺ ions is often used at between 500eV and 5keV. In addition to the survey scan of the elemental composition of the uppermost surface and the measurements of the relative atom concentrations of the elements in the scanned region, an AES method using sputtering provides quantitative compositional information about the distribution of the

elements with depth below the sample surface as a function of sputter time and thus determines their thickness.

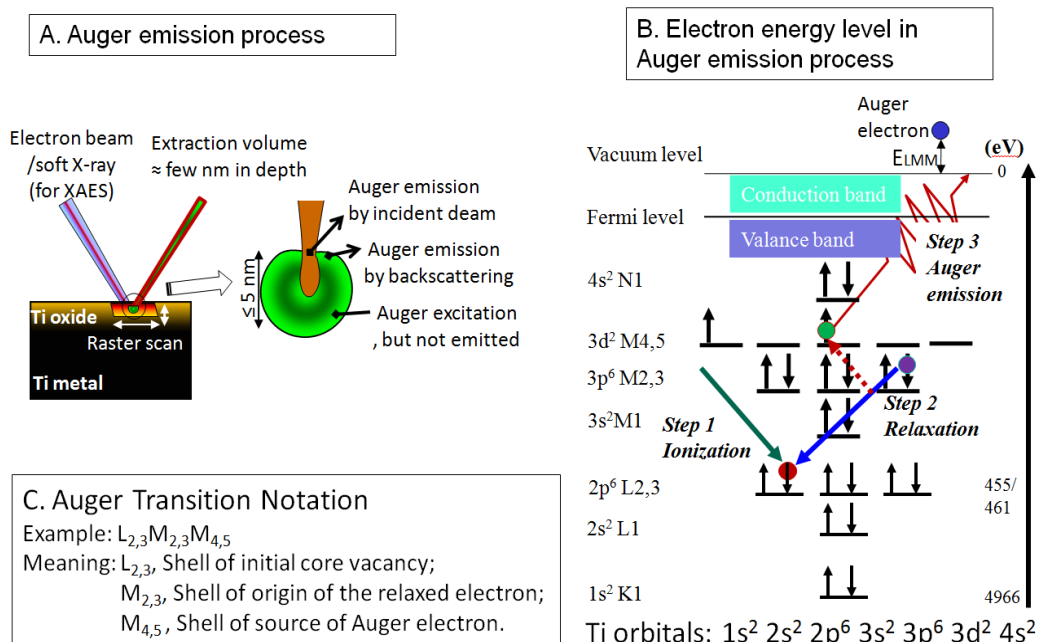


Figure 3–13. The cartoon shows A) the Auger emission process, B) the electron configuration and energy levels of the titanium atom and the Auger emission steps, as follows: the first step – ionization, the second step – relaxation, and the third step – auger emission, and C) an example of Auger transition notation. The LMM Auger electron energy in use of monochromated Al K α is ≈ 1065 eV. $E_{LMM\text{ Auger}} = E_{L_{2,3}} - (E_{M_{2,3}} + E_{M_{4,5}})$. (For details, see text).

Table 3–2. X-ray and spectroscopic notation

Quantum number	X-ray	X-ray	Subshell	Spectroscopic
n L j	suffix	level		level
1 0 1/2	1	K	s	1s1/2
2 0 1/2	1	L1		2s1/2
2 1 1/2	2	L2	s, p	2p1/2
2 1 3/2	3	L3		2p3/2
3 0 1/2	1	M1		3s1/2
3 1 1/2	2	M2		3p1/2
3 1 3/2	3	M3	s, p, d	3p3/2
3 2 3/2	4	M4		3d3/2
3 2 5/2	5	M5		3d5/2

The electron shells are labelled K, L, M, N, O, P, and Q, which is designated $n = 1, 2, 3, 4, 5, 6$, and 7 .

n = Principal quantum number, can have values $1, 2, 3, 4, \dots$

l = Orbital angular quantum number, can have values $0, 1, 2, 3, 4, \dots$

j = Total angular momentum of one specific electron. (Adapted from reference 32).

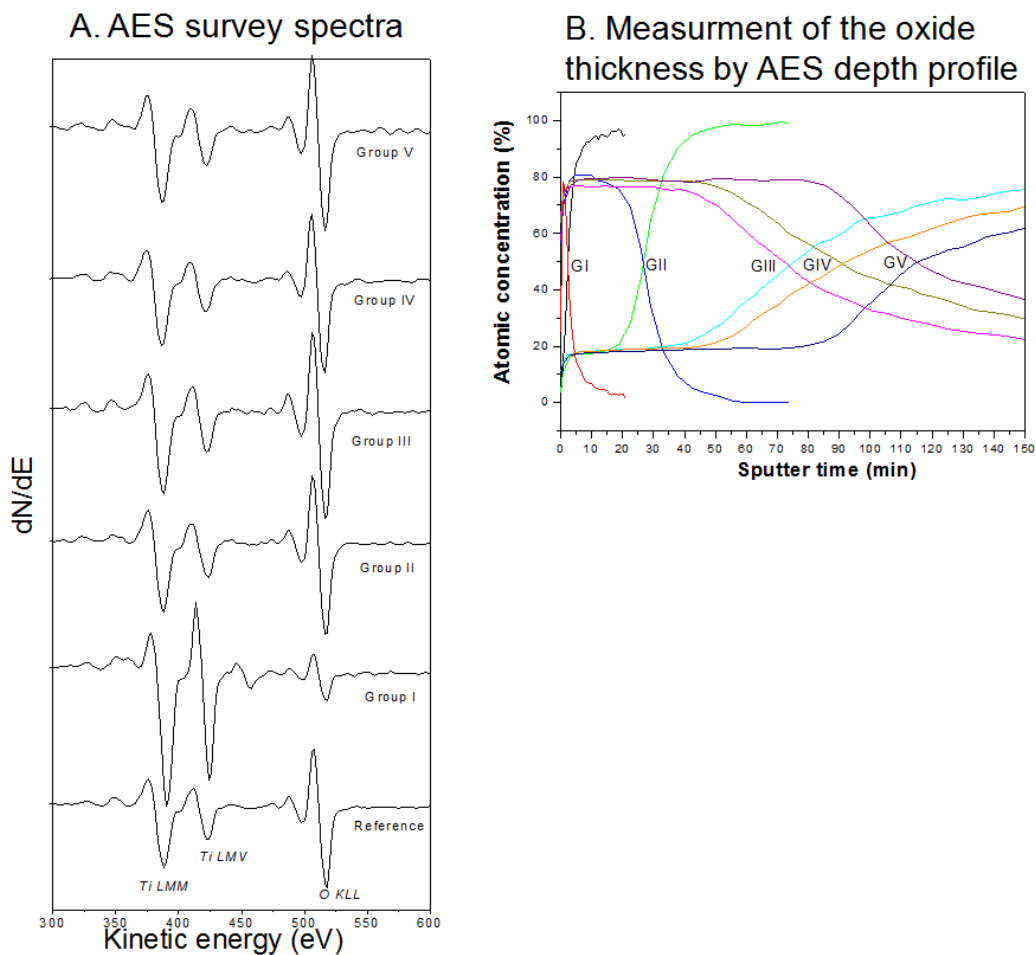


Figure 3–14. A) AES survey spectra show the peaks of Ti LMM and Ti LMV, a peak of O KLL of a reference material, the machine-turned implant (G I), oxidized titanium implants (G II – V), B) AES depth profiles presenting oxide thickness of ≈ 17 nm (G I) for a native, thermal oxide and approximately 200–1000 nm (G II – V) for the anodized titanium implants. [6] The reference TiO₂ film was prepared on Si (1 0 0) by metal organic chemical vapor deposition. The reference oxide film was characterized as 91.8 nm thick, a refractive index of 2.199, as evaluated by ellipsometry. The sputter rate is approximately 7.1 nm/min.

3.3.4.3. X-ray Photoelectron Spectroscopy (XPS)

Together with AES, XPS, also known as ESCA (Electron Spectroscopy for Chemical Analysis), is a surface sensitive spectroscopic technique that is commonly used to investigate the surface chemistry of dental implants. The XPS technique undertakes the major functions needed to characterize the surface chemistry of dental implants, such as the following: i) all elemental compositions (atomic number, $Z \geq 3$, except hydrogen and helium) and their relative atomic concentrations in the surface region of interest (the uppermost 1–10 nm usually), ii) chemical bonding or electronic state of each element in the surface, iii) elemental distribution across the topmost surface line (line profiling or mapping), and iv) depth distribution of elemental composition and thus the thickness of a thin film as a function of ion beam etching time (depth profiling). The physical principle of XPS is based on Einstein's photoelectric effect: $E = h\nu$, where h = Planck constant (6.62×10^{-34} Js) and ν = frequency (Hz) of the radiation. The photoelectron emission process in an XPS system can be divided

into two steps: 1) X-ray photon irradiation and consequent ionization of the atoms in the sample surface (photoionization) and 2) the creation and escape of the photoelectron (photoelectron emission). When a focused beam of X-rays with energy $h\nu$ through a system of electron collection lenses is irradiated onto the sample surface mounted in an ultra-high vacuum (UHV) condition (Figure 3–15 A), photoelectrons are ejected with kinetic energy from the electron shells (either the core electrons or the outmost valance electrons) of the surface atom that the irradiated x-ray collides with (Figures 3–9D and 3–15B). The electron binding energy of an emitted photoelectron can be calculated by the following equation:

$$E_B = h\nu - E_k - \phi$$

where E_B = the binding energy (BE) of the core electron, $h\nu$ = the kinetic energy of incident X-ray photon, E_k = the kinetic energy of emitted photoelectron as measured by the electron energy analyzer, and $\Phi = (E_{\text{vac}} - E_{\text{Fermi}})$, the work function of the instrument. For the photoelectrons to escape through the electron paths, the energy of photoelectrons, E_k , should be ejected with more kinetic energy than binding energy, E_B . The electron energy analyzer in an XPS system simultaneously detects the kinetic energy and the quantity of the emitted photoelectrons. Thus, XPS spectral lines are identified as characteristic kinetic energies of the shell electrons that were ejected from the surface atoms of interest.

For a better understanding of surface chemistry characterization by XPS, consider the basic physical principle that defines the energy level of an electron in the surface atom (Figure 3–15 B). An electron, as a “charged particle and wave,” travels around the nucleus along certain distinct orbitals (electron shells) in an atom, which in turn lead to an angular momentum and a spin momentum. The energy level of the electron is mainly determined by the type and level of an orbital, taking into account the energy level transitions in the order of increasing energy, 1s, 2s, 2p, 3s, 3p, 4s, 3d, 4p, 5s, 4d, 5p, 6s, 4f, 5d, 6p, 7s, 5f, and so on, as well as spin-orbit coupling, i.e., the interaction of an electron's spin magnetic moment with its motion magnetic moment in a given orbital. The angular momentum of the electron is the sum of orbital angular momentum and spin momentum. Thus, the specific energies of the electrons of an atom differ from atom to atom and also vary with the *specific bonding states* of the atoms that constitute chemical substances. Briefly, an electron of an atom imposes inherent, characteristic energy levels, which are changed as the atoms form chemical substances that contain two or more atoms. This change of the chemical bonding state produces changes in the binding energy of a core electron of the atoms. It is called a *chemical shift*. Chemical shift information empowers the XPS method to characterize the functional group, the chemical bonding state, and the oxidation state of the metals such as the titanium in dental implants (see the examples below). By measuring the characteristic binding energies and the chemical shift (the deviation of binding energy relative to the known binding energy) of the photoelectrons that originate from the atoms, an XPS system is able to directly identify and quantify the atoms and also to measure the specific bonding and electronic states of the atoms that constitute chemical substances. It is important to note that the photoelectrons that are extracted and therefore detected in an electron energy analyzer only originate from the topmost surface in a depth of ≤ 10 nm (Note: the extraction volume is distinguished from the excitation volume, see Figures 3–13A and 3–15 A), depending on the specifications of the commercial products. The other photoelectrons that are excited in a deeper volume are either recaptured or trapped within the volume and therefore are not detected. This activity may

explain how XPS analyses are able to characterize the surface chemistry of the implant sample and why XPS is a non-destructive surface sensitive technique and has perhaps the most powerful benefits for surface chemistry characterization over other methods.

In the XPS nomenclature of j - j coupling, the principal quantum number appears first, then the subshell letter follows, and the total angular momentum is finally appended. The principal quantum number, n can take 1, 2, 3, 4, 5. . . . The subshells of an electron are labelled $s, p, d, f, g, \dots$. The $n = 1$ state has one subshell, called $1s$; the $n = 2$ state has two subshells, called $2s$ and $2p$; the $n = 3$ state has $3s, 3p$, and $3d$; and so on. For instance, titanium has the electron configuration of $1s^2 2s^2 2p^6 3s^2 3p^6 3d^2 4s^2$. The total angular momentum of one particular electron j is presented in a combination of orbital angular momentum and spin momentum. The orbital angular quantum number l can take the values of 0, 1, 2, 3, 4, 5, . . . which correspond to the letters, $s, p, d, f, g, \dots$, respectively. The spin quantum number s can take on the values $+\frac{1}{2}$ or $-\frac{1}{2}$. Thus, the total angular momentum can be either $j = l + s$ or $j = l - s$, which gives the j values, $1/2, 3/2, 5/2, 7/2$, and so on. Take an example, $2p_{3/2}$ of titanium (Figure 3–15C), where “2” is the principal quantum number, “p” designates the p subshell in the L shell, the suffix $3/2$ denotes the total angular momentum, j . Some of the quantum numbers and electron shells used in XPS notation are listed in Table 3–2.

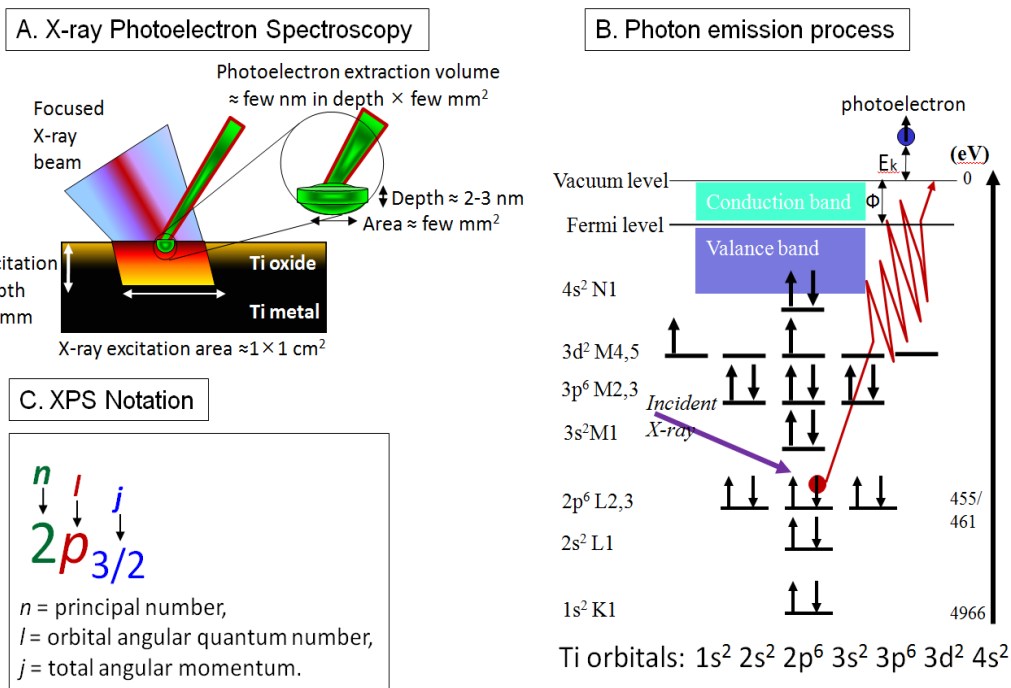


Figure 3–15. The cartoon shows A) X-ray beam excitation can be performed in the area $\approx 1 \times 1 \text{ cm}^2$ and the penetration depth $\approx 1 \text{ mm}$. However, photoelectron can be generated only from a narrow extraction volume $\approx$ few nm in depth $\times$ few mm^2 because of a limit of their escape depth. B) schematic diagram of the photon emission process exemplified in titanium. $E_k = h\nu - E_B - \Phi$, where E_k = kinetic energy of emitted photoelectron, $h\nu$ = energy of X-ray photon, h = Plank's constant ($6.62 \times 10^{-34} \text{ Js}$), ν - frequency (Hz) of the radiation, E_B = binding energy of core level, and Φ = work function of the instrument. For titanium, the X-ray photon energy at Ti 2p is $\approx 457 \text{ eV}$ ($E_{h\nu} = E_{L2} - E_{M4}$), and C) an example of XPS nomenclature.

Table 3–3. Relative atom concentration at Ti2p, O1s, Mg2p, Ca2p, P2p, F1s, Na1s, Cl2p, C1s, and N1s, acquired from the as-received surfaces of machine-turned (a type of Brånemark implant), Osseotite[®], Osseospeed[®], SLA[®], SLActive[®] TiUnite[®], calcium-incorporated Ospanol[®], and magnesium-incorporated implant M[®]

Elements	c.p. titanium	Osseotite	OsseoSpeed	SLA
Ti	16,0	16,7	11,8	20,1
O	39,3	38,7	33,1	47,1
Mg	-	-	-	-
Ca	0,3	-	1,3	-
P	-	-	-	-
F	-	-	0,3	-
Na	-	-	-	-
Cl	-	-	-	-
C	43,2	44,0	53,2	32,0
N	1,2	0,6	0,3	1,0
Elements	SLActive	TiUnite	Ca implant	Mg implant
Ti	8,4	12,0	15,6	9,9
O	24,1	45,2	47,6	50,1
Mg	-	-	-	6,8
Ca	-	-	3,8	-
P	-	6,8	-	5,2
F	-	-	0,6	-
Na	16,1	-	-	-
Cl	15,8	-	-	-
C	35,6	35,1	30,0	26,1
N	-	1,0	2,4	1,9

On the basis of the principle mentioned above, most of the XPS system is instrumented to effectively detect the emitted photoelectrons and displays two types of spectra, i.e., a wide-scan survey spectrum and a high-energy resolution spectrum of an interest element. The survey scan enables us to identify all elements and to quantify the amount, relative atomic concentration, at.%, while the high-resolution scan provides information on chemical bonding and electronic states. To generate the photoelectron, the commonly used x-ray sources are a monochromated Mg K α radiation (KE = 1253.6 eV, line width = 0.7 eV) and Al K α radiation (KE = 1486.6 eV, line width = 0.85 eV). The emitted photoelectrons have kinetic energies in the range of ≈ 0 - 1250 eV or ≈ 0 - 1480 eV, respectively. A typical XPS spectrum is obtained by plotting the number/amount of photoelectrons detected (Y-axis) versus the binding energy of the electrons detected (X-axis).

For interpretation of the measured XPS spectra, the process involves *peak position*, *intensity*, full width at half maximum (FWHM), peak area measurement, resolution enhancement, curve fitting, and deconvolution. A number of database libraries for the binding energies and peak shapes of most known elements and their compounds are provided for the interpretation of XPS spectra in various manufacturer manuals, handbooks and websites. [26-35] Peak assignments of unknown elements can be performed by comparisons to binding

energies of most known materials. Prior to XPS data interpretation, however, it is important to determine whether the as-received survey spectra have or have not been shifted primarily due to surface charging. The survey spectra should be, if necessary, calibrated with reference XPS lines. In general, the carbon 1s peaks near 284.8 ± 0.2 eV binding energy are used as a calibration reference. Alternatively, the spectrometer can be calibrated with the binding energy of the electron shell and the spin-orbit of a well known element. Figure 3–16 shows a typical XPS wide-scan spectrum obtained from the titanium oxide surfaces of screw-shaped dental implants including machine-turned (a type of Brånemark implant), Osseotite[®], Osseospeed[®], SLA[®], SLActive[®] TiUnite[®], calcium-incorporated Ospan[®], and magnesium-incorporated implant M[®].

To calculate relative atomic concentrations, at.%, one can use the atomic sensitivity factor (ASF), which divides the number of the photoelectrons detected, and one can use a standard reference element measured on the same instrument for calibration. For quantification of the XPS spectra, it is assumed that the number of the detected photoelectrons is proportional to the number of atoms in a given state. Table 3–3 shows binding energies (BEs) that identify the shell and spin-orbits of each peak of the elements, Ti2p, O1s, Mg2p, Ca2p, P2p, F1s, Na1s, Cl2p, C1s, and N1s, acquired from the as-received surfaces of machine-turned (a type of Brånemark implant), Osseotite[®], Osseospeed[®], SLA[®], SLActive[®] TiUnite[®], calcium-incorporated Ospan[®], and magnesium-incorporated implant M[®]. The Ar ion beam sputter cleaning was carried out for three seconds (beam energy, 2KeV; primary current, 2 μ A; raster area, 3.14mm²), which is evaluated to remove the superficial contaminants of two monolayers (1 monolayer $\approx$ 0.3 nm).

The interpretation of XPS spectra is not always straightforward, particularly in the case that the photoelectron emission lines make complex, overlapping peak shapes. For the more precise identification of intensity, position, and shape of the measured peaks, a number of fitting techniques can be used. The deconvolution process is a technique that resolves or decomposes the overlapping peaks into a set of separated single peaks of which positions are known and extracts the underlying single peak shapes from the broadened peaks. The deconvolution technique is especially useful for identifications of functional groups and chemical bonding states of the elements. As shown in Figure 3–17, the peak shapes of the XPS spectra are very much dependent on the chemical states of the surface analyzed. The machined-turned, blasted, etched titanium implants involving Brånemark implants, Osseotite[®], Osseospeed[®], and SLA[®] reveal relatively simple peaks of their XPS spectra, because the chemical compositions of those implant surfaces consist simply of TiO₂ and nonstoichiometries of TiO such as Ti₂O₃, and always contain contaminants of carbon species due to air exposure and some impurities (Figure 3–16). Unlikely, the oxidized implants such as TiUnite[®], calcium-incorporated and magnesium-incorporated implants present more complicated peak shapes in the XPS spectra, because P anions in the case of Tiunite[®], Ca and Mg cations are incorporated into surface titanium oxide of the implants, by surface engineering processes, leading to more complicated surface chemistry (Figure 3–17).

Deconvolution data in Figure 3–18 show overlapping and broadening of Ti 2p and O 1s core-level energy regions corresponding to the wide scan and high-resolution spectra (Figures 3–16 and 3–17) of the titanium oxides of screw-shaped dental implants including machine-turned (a type of Brånemark implant), Osseotite[®], Osseospeed[®], SLA[®], TiUnite[®], and calcium-incorporated and magnesium-incorporated implants.

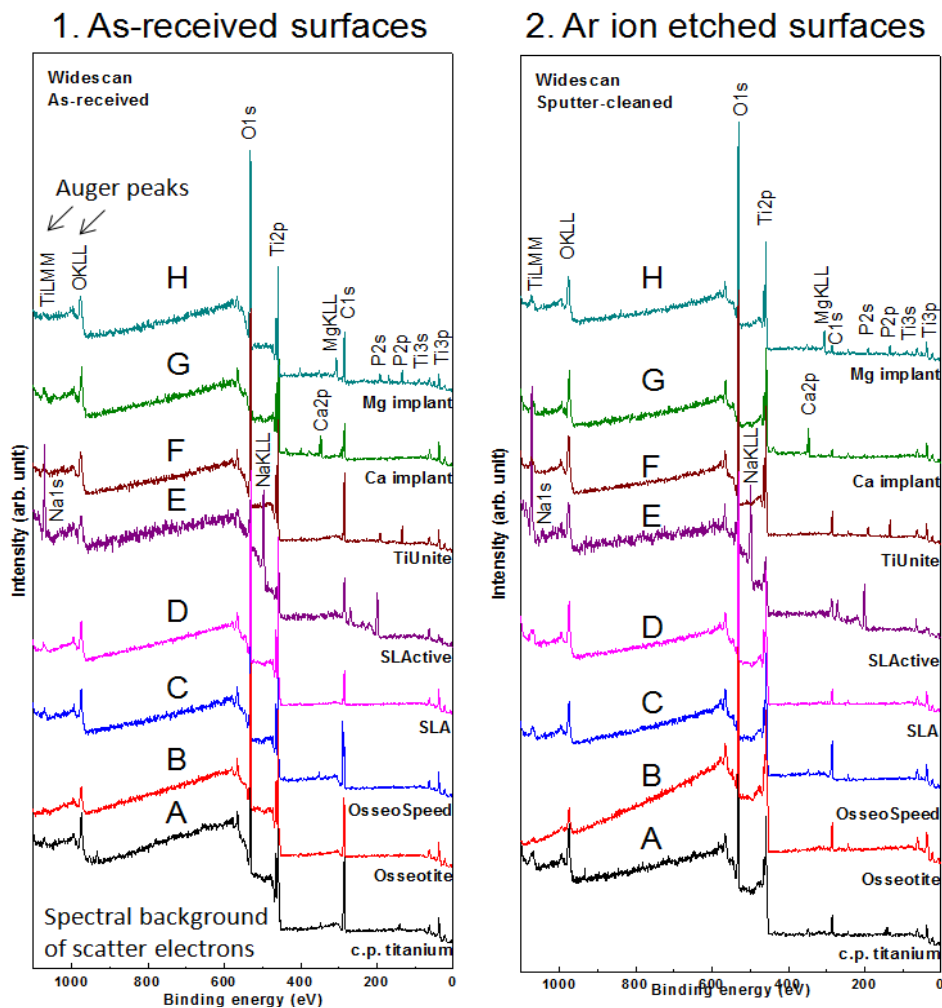


Figure 3–16. Typical XPS survey spectra of 1) as-received and 2) after Ar ion beam sputter cleaned surfaces of A) machine-turned Brånemark implant, B) dual acid etched Osseotite®, C) blasted and acid etched Osseospeed®, D) blasted and acid etched SLA®, E) blasted and acid etched, soaked SLActive®, F) oxidized, phosphorous-incorporated TiUnite®, G) oxidized, calcium-incorporated Ospan®, and H) oxidized, magnesium-incorporated implant M®. The spectra were obtained using a monochromatic Al K α X-ray source (1486.7 eV, 300 W; the beam size, 400 μ m diameter, the electron take-off angle at 45°, the vacuum pressure below 10^{-9} torr. Ar sputter cleaning was carried out for 3 s (beam energy, 2 KeV; primary current, 2 μ A; raster area, 3.14 mm 2), resulting in a removal of the superficial contaminants (mainly carbon species) of approximately 0.6 nm (two monolayers). XPS spectra are plotted as a function of the binding energy with a resolution of 0.1 eV, calibrated with the carbon 1s peaks near 284.8 ± 0.2 eV.

Detailed characterization reveals that the binding energies of $2p_{1/2}$ and $2p_{3/2}$ of Ti metal are ≈ 459.9 eV and ≈ 453.7 eV, respectively. The difference of binding energy between $2p_{1/2}$ and $2p_{3/2}$, i.e., the chemical shift ($\Delta BE = BE_{2p_{1/2}} - BE_{2p_{3/2}}$), is $\Delta eV = 6.2$ eV. In the case of TiO_2 , the binding energies of $2p_{1/2}$ and $2p_{3/2}$ are ≈ 464.4 eV and 458.7 eV, respectively. The difference of binding energy between $2p_{1/2}$ and $2p_{3/2}$, i.e., the chemical shift ($\Delta BE = BE_{2p_{1/2}} - BE_{2p_{3/2}}$) is $\Delta eV = 5.7$ eV. As a result of the comparisons between Ti metal and TiO_2 , it is clearly found that the binding energies of $2p_{1/2}$ and $2p_{3/2}$ of the Ti metal are lower than the binding energies of titanium oxides, whereas the difference in the chemical shift between

$2p_{1/2}$ and $2p_{3/2}$ is bigger at 2p of Ti metal than at 2p of TiO_2 . A major reason for the differences of the binding energies and chemical shift at $2p_{1/2}$ and $2p_{3/2}$ between Ti metal and TiO_2 may be explained as follows. The orbital electrons of the titanium atoms tend to be withdrawn as Ti is ionized to Ti^{+4} as in TiO_2 , by a polarization of the valence shells due to the strong electronegativity of the oxygen atoms in the TiO_2 . Hence, electrostatic interactions (attraction force) of the Ti nucleus by the outer shell electrons of the 2p orbitals of the titanium atom, i.e., $3s^2 3p^6 3d^2 4s^2$, and the electronic interactions between the core orbital electrons become weakened in energy. Thus, $2p_{1/2}$ and $2p_{3/2}$ of TiO_2 relaxes to a relatively high-binding energy as compared the case of Ti metal. The same principle can explain why the chemical shift between $2p_{1/2}$ and $2p_{3/2}$ becomes lower at 2p of TiO_2 metal than that of Ti metal. In addition to chemical state identification, XPS is also utilized to measure the thin oxide thickness of titanium dental implants as AES measurements (See the oxide thickness section below).

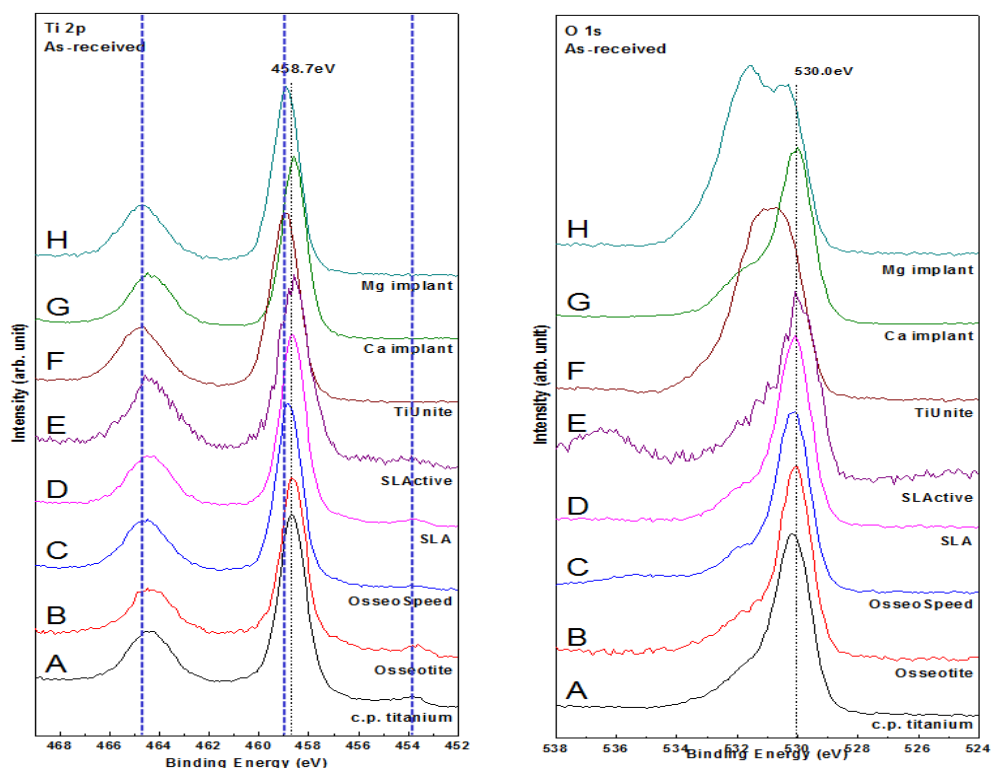


Figure 3-17. High-resolution XPS spectra at Ti 2p and O 1s of 1) as-received and 2) after Ar ion beam sputter cleaned surfaces of A) machine-turned Brånemark implant, B) dual acid etched Osseotite[®], C) blasted and acid etched Osseospeed[®], D) blasted and acid etched SLA[®], E) blasted and acid etched, soaked SLActive[®], F) oxidized, phosphorous-incorporated TiUnite[®], G) oxidized, calcium-incorporated Ospol[®], and H) oxidized, magnesium-incorporated implant M[®]. The spectra show complex, overlapping-peak shapes of the photoelectron emission lines due to chemical shift, indicating differences of surface chemistry/chemical binding states between the implants. To identify the differences of surface chemistry between the implants, the spectra should be deconvoluted to resolve or decompose the overlapping peaks into a set of separated single peaks. (See Figure 3-18).

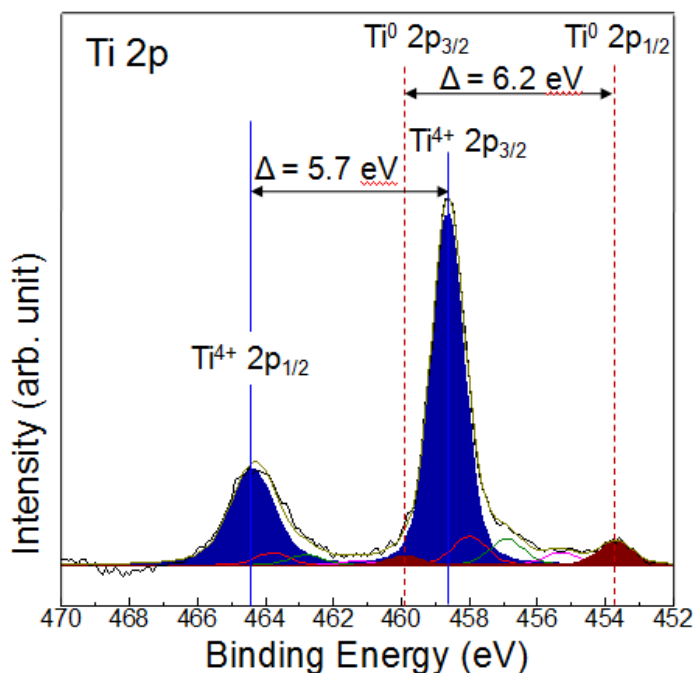


Figure 3–18. High-resolution spectra are deconvoluted at Ti $2p_{1/2}$ and Ti $2p_{3/2}$ core-level binding energy of both Ti metal (Ti^0) and TiO_2 (Ti^{4+}). A deconvolution process was performed using a numerical fitting procedure with combinations of the Lorentzian and Gaussian functions, enabling precise identification of intensity, position and shape of the measured peaks. The chemical shift ($\Delta \text{BE} = \text{BE } 2p_{1/2} - \text{BE } 2p_{3/2}$) is $\Delta \text{eV} \approx 6.2 \text{ eV}$ for Ti metal (Ti^0) and $\Delta \text{eV} \approx 5.7 \text{ eV}$ for TiO_2 (Ti^{4+}). (For details, see text).

Importantly, it should be noted that the surface chemistry changes through the anions incorporation of phosphorus for TiUnite[®] or the cations incorporation of calcium or magnesium into the thick oxide layer for Ospan[®] and Implant M[®], respectively (see the surface chemistry section below). Sometimes, the evidence that surface chemistry is changed has been neglected in the literature on effects of the surface properties of TiUnite[®] *in vitro* and *in vivo*. This case is another case of “misleading perceptions” about the surface properties, showing that the role of surface chemistry might have been incorrectly interpreted in the literature due to poor surface characterization. Still, with deep understandings of characterization and the properties of the implant surfaces, caution must be taken when interpreting the effects of the surface properties on implant performance *in vivo* and *in vitro*. For more information about AES and XPS, the interested reader is referred to a number of useful books [25–31] and website resources [32–41]. Freely accessible useful online databases for AEA and XPS spectral interpretation are introduced [42–50].

3.3.5. Oxide Thickness

Oxide thickness can usually be determined by microscopic and spectroscopic measurements. The former measurement is direct, yielding precise thickness values, and the latter is indirect, leading to some deviations in the values. As shown in Figure 3–4, the SEM images in the cross-sectional view show that blasted, etched, and blasted/etched implants,

including Tioblast[®], Osseotite[®], and SLA[®], bear very thin oxide layers that are hardly detectable by the direct measurement technique of SEM, while indirect methods, such as spectroscopic measurements, can be utilized in cases of such thin oxide thicknesses of approximately 3-5 nm [21]. Instead, in the case of a thick oxide over a few tens of nanometers, the SEM method takes direct and precise measurements on a cross-sectional sample. In general, surface preparation for microscopic examination of cross-sections can be performed by several methods, including a metallurgical cutting-grinding-polishing procedure, fracture techniques, ultramicrotome section, or an ion/focused ion beam (FIB) beam milling process. The present cross-sections were prepared following nickel (Ni) plating for preventing an oxide layer on the implant surface, resin embedding, grinding and polishing (Figures 3–8b and 3–11). The SEM method is capable of measuring the oxide thickness of electrochemically oxidized implants such as TiUnite[®], Ospol[®] and Implant M[®], showing the oxide thickness in the range of 3 - 10 μm . A thin oxide layer, such as air-formed native oxides less than a few nanometers, is not readily detectable by the SEM method due mainly to difficulties in routine quality control of cross-section preparations. Alternatively, the depth profiling techniques in AES and XPS are favored for measuring a thin film oxide thickness. Using this Auger depth profiling technique, Figure 3–14b shows the depth distributions of Ti and O elements of the thin and thick surface oxides of machine-turned and anodized titanium implants. Oxide thickness can be calculated by calibrating a sputter rate of AES in use in association with a reference material of known thickness. The determination of the oxide thickness was calculated by the numerical formula $d = v_0 t_d$, where d is the film thickness, v_0 is the sputter rate, and t_d is the sputter time to make the oxygen peak amplitude decrease by 50% [19]. This measurement is an indirect measurement of the oxide thickness. Several factors that affect variation of the oxide thickness are related to the AES depth profiling technique such as uneven sputtering and are also related to the screw-shape implants such as the thread angle that requires a tilting of the sample for angle adjustment to the probing beam (Figure 3–7). These concerns lead to difficulties in the measurement of a precise thickness.

Another method of XPS measurement of the oxide thickness is based on the fact that the binding energies differ between the metal oxides and the metallic species (Δ BE). In the case of titanium oxide, 2p of Ti and TiO_2 show the difference in the binding energy, as mentioned above. The equation used to determine the SiO_2 thickness by XPS [51] can be adapted to be similar to the following equation:

$$d = \lambda_{\text{TiO}_2} \sin\theta \ln \left[\left(\frac{1}{\beta} \right) \left(\frac{I_{\text{TiO}_2}}{I_{\text{Ti}}} \right) + 1 \right]$$

where λ is the attenuation length of the Ti 2p photoelectrons in TiO_2 , θ is the angle between the sample surface plane and the electron analyzer, β is a sample-dependent constant (the ratio of the Ti 2p intensity from infinitely thick TiO_2 and Ti), I_{TiO_2} is the intensity (peak area) of TiO_2 , and I_{Ti} is the intensity (peak area) of Ti. The ratio ($I_{\text{TiO}_2}/I_{\text{Ti}}$) of the intensities (peak areas) of the TiO_2 and Ti photoelectron peaks is a determining parameter. Many factors affect spectroscopic depth profiling, including instrument parameters, experimental conditions, and characteristics of the sample surface. In fact, the screw geometry, roughness, crystal structure, and surface charging are particularly important variables related to dental implants. In addition to SEM, AES and XPS described here, oxide thickness can be determined by secondary ion mass spectrometry (SIMS), Rutherford backscattering (RBC) and transmission

electron microscopy (TEM). Like the SEM method, TEM is able to directly measure a thin oxide thickness, thus providing the only “true” measurement for a high resolution cross-section, although the cross-sectional preparation of the titanium sample for TEM is very difficult.

Conclusion

Unlike over the last three decades, in which titanium dental implants have been dominated by moderately rough microstructured surfaces, recently emerging micro- and nanotechnology is rapidly advancing surface engineering in implant dentistry. Such advances in surface engineering technologies have resulted in more complicated surface properties at the micro- and nanometer scale, including the structure/topography, chemistry, crystal structure, and physical and mechanical properties. This situation necessitates comprehensive surface characterizations of titanium dental implants. With inadequate understanding and/or poor surface characterizations, the roles of surface properties in performance (both *in vivo* and *in vitro*) might have been incorrectly interpreted in the literature. Still, caution must be taken with deep understanding of characterization methods and resultant surface properties when interpreting the effects of the surface properties on implant performance. A better understanding of the principles and applications of surface characterization methods and resultant surface properties will provide benefits for advancing our knowledge about implant surfaces and, furthermore, will enhance our understanding with respect to the role of implant surfaces in osseointegration. Ultimately, such knowledge and understanding will greatly contribute to the development of smart dental implant surfaces. There is no gold standard with respect to what surface properties of dental implants must be evaluated and what surface characterization methods should be undertaken for them. It is likely that surface properties can be better characterized by a combination of techniques in such a way as to complement the limitations and strengths of each method compared to using a single method alone.

Acknowledgment

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Part III – In Vitro Models in Dental Implant Research

Chapter IV

Use of Simulated Body Fluid for Predicting the *In Vivo* Bone Bioactivity of a Material

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4.1. Introduction

The stable fixation of dental implants over an extended period of time is an important challenge in dentistry. The stability of dental implants is largely affected by the structure at the interface of the implant with the surrounding bone. Generally, synthetic materials implanted into bone defects are encapsulated by a fibrous tissue so as to be isolated from the surrounding bone. Titanium and its alloys are unique in their ability to come into direct contact with the surrounding bone when the surface of these materials is made micro-rough by grit blasting or acid etching. [1] Therefore, they are commonly used as dental implants with roughened surfaces. [2] However, this direct contact does not necessarily entail actual bonding between the implant and the bone. Such actual bonding at the interface would be desirable for stable fixation of the implants over a long period of time. There are certain kinds of ceramic materials that can bond tightly to living bone, and these are called bioactive ceramics [3]. However, they cannot be used as dental implants because of their low level of fracture resistance. If it were possible to identify *in vitro* which materials can bond to living bone and thus are suitable as dental implants, it would greatly aid in the discovery of such materials.

In 1972, Hench et al. found that certain glasses in the system $\text{Na}_2\text{O}-\text{CaO}-\text{SiO}_2-\text{P}_2\text{O}_5$ spontaneously bonded to living bone without the formation of surrounding fibrous tissues [4] and called them collectively “Bioglass.” Subsequently, several types of ceramics, such as sintered hydroxyapatite [5], sintered β -tricalcium phosphate (β -TCP) [6], apatite/ β -TCP biphasic ceramic [7], and glass-ceramic A-W containing crystalline apatite and wollastonite

[8] have been also found to bond to living bone. These ceramics are in clinical use and have an important role as bone substitutes.

Most of these materials form a bonelike apatite layer on the material surface in the living body and form a bond with living bone through the apatite layer [9]. In general, a synthetic material forms a bone-like, apatite layer on its surface in the living body and is able to form bond to living bone. The *in vivo* apatite formation on these bone-bonding ceramics was found to be reproducible in an acellular simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma [9]. This indicates that the bone-bonding bioactivity of a material can be predicted from the apatite formation on its surface in SBF. On the basis of these findings, the bone-bonding abilities of various kinds of materials have been examined using SBF prior to animal experiments by many researchers. After some modifications [10], SBF was standardized as a solution for the *in vitro* evaluation of the apatite-forming ability of implant material by the International Standard Organization in 2007.

The history of SBF and validity of the SBF test were reviewed by the present authors in 2006 [9]. Some comments taking exception to this review paper were later published by Bohner et al. [11] and Pan et al. [12]. Bohner et al. pointed out that the bone-bonding ability of certain resorbable ceramics cannot be evaluated by SBF. We agree, and this was already mentioned in our review paper in 2006 [9]. He also pointed out that a different protocol for preparing SBF can be formulated. This is true. However, any such protocol must be examined by its stability and the suitability of the solution prepared by the new protocol for standardization.

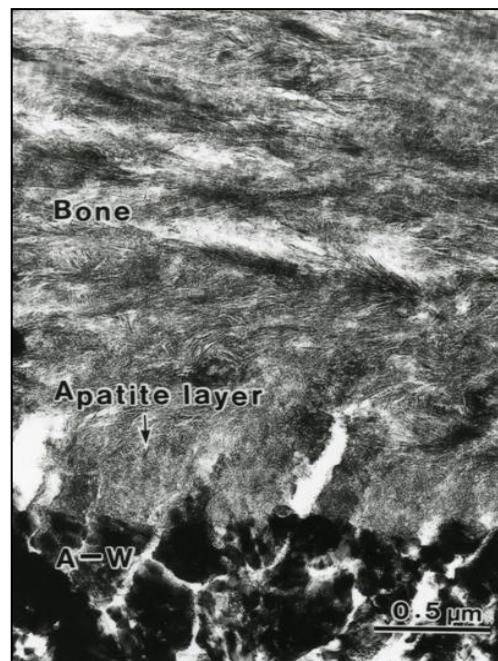
Pan et al. pointed out that apatite-forming ability is not necessarily a predictor of bone-bonding ability. They used borosilicate glass as an example, which forms apatite on its surface in SBF but does not bond to living bone. It should be noted here that the boron released from the borosilicate glass can exhibit cytotoxicity. It was mentioned in our review paper in 2006 [9] that a material that contains a cytotoxic or antigenic component is not likely to bond to living bone, even if it forms the apatite on its surface.

The usefulness of the SBF test should not be overturned because of these few exceptional cases. Of course, the bone-bonding ability of any material must ultimately be tested by animal experiment. However, if the SBF test is applied keeping these exceptional cases in mind, it is indeed a useful method for selecting a candidate bone-bonding material prior to animal experiment. The bone-bonding ability of glasses, glass-ceramics, sol-gel derived materials, metals, etc., vary largely according to their composition, as well as heat and surface treatments, etc. If the bone-bonding abilities of these materials are directly examined by animal experiments, an unmanageably large number of animal experiments would be required. By using the SBF test, the cost and term of the animal experiments, as well as the number of animals needed for sacrifice, can all be substantially reduced. In the present chapter, the background and procedures of the SBF test are reviewed.

4.2. Background of the SBF Test

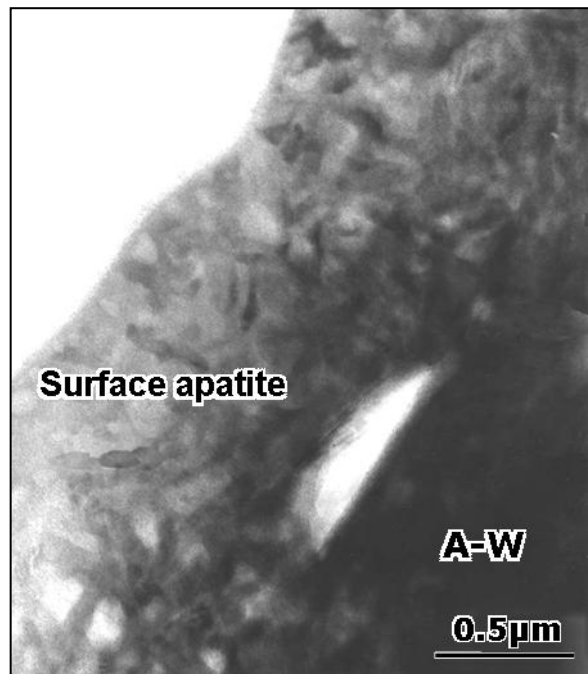
In 1990, it was found that the glass-ceramic A-W precipitating crystalline apatite and wollastonite in an MgO-CaO-SiO_2 glassy matrix forms an apatite layer on its surface in the living body after implantation and bonds to living bone through the apatite layer, as shown in

Figure 4-1 [13,14]. However, the glass-ceramic A-W(Al), which also contains precipitating crystalline apatite and wollastonite, but in an Al_2O_3 -containing MgO-CaO-SiO_2 glassy matrix, neither formed the apatite on its surface in the living body nor bonded to living bone [15]. This showed that the apatite layer that formed on the glass-ceramic A-W plays an important role in forming the bond of glass-ceramic A-W with living bone. A CaO-SiO_2 glass, [16] Bioglass, [4] $\text{Na}_2\text{O-CaO-B}_2\text{O}_3\text{-Al}_2\text{O}_3\text{-SiO}_2\text{-P}_2\text{O}_5$ glasses, [17] Ceravital-type glass-ceramic precipitating crystalline apatite [18], Bioverite-type glass-ceramic precipitating apatite and phlogopite [19], sintered hydroxyapatite [20], and Hydroxyapatite/beta tricalcium phosphate (β -TCP) biphasic ceramics [21] were also found to form a surface layer of apatite or calcium phosphate layer in the living body and to bond to living bone thereby. It appears from these results that the essential requirement for synthetic material to bond to living bone is the formation of an apatite layer on its surface in the living body. However, these are materials such as calcium sulfate that form apatite on their surface in the living body but are replaced with living bone instead of bonding to living bone [22]. These materials also can be seen as a kind of bone-bonding material. Natural abalone shell does not bond to living bone, although it forms an apatite layer on its surface in the living body [23, 24]. This is attributed to an antibody reaction to the proteins contained in the abalone shell. β -TCP [25] and calcite (CaCO_3) [26] bond to living bone without forming any detectable apatite on their surfaces. This is attributed to their high resorption rate in the living body. Therefore, it can be said that a material that forms apatite on its surface in the living body bonds to living bone, insofar as it contains neither a toxic nor antigenic component, although some resorbable materials are replaced with living bone after forming the apatite on their surfaces or bond to living bone without forming any detectable apatite on their surfaces.



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Figure 4.1. Transmission electron micrograph of an interface between glass-ceramic A-W and rat tibia [14].



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Figure 4.2. Cross-section of apatite layer formed on glass-ceramic A-W in SBF [27].

The apatite formation on the glass-ceramic A-W [27], a CaO–SiO₂ glass [28], Bioglass [29], the Na₂O–CaO–B₂O₃–Al₂O₃–SiO₂–P₂O₅ glasses [17], Ceravital-type glass-ceramic [18], sintered hydroxyapatite [30,31], abalone shell [23,24] and calcium sulfate [32] was reproduced on their surfaces in the acellular simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma [33,34], as shown in Figure 4–2. However, the glass-ceramic A-W (Al) did not form the apatite on its surface even in SBF, similar to the result *in vivo* [35]. This indicates that the apatite formation on the surface of the synthetic materials in the living body is not governed by cellular reactions but by the chemical reaction of inorganic ions in the body fluid. According to the analysis of the apatite formation on a sintered hydroxyapatite in a serum-containing SBF, the apatite formation on its surface is slightly decreased in rate but otherwise is not disturbed by the presence of the proteins [36]. This might be attributed to the high degree of supersaturation of the body fluid with respect to the apatite.

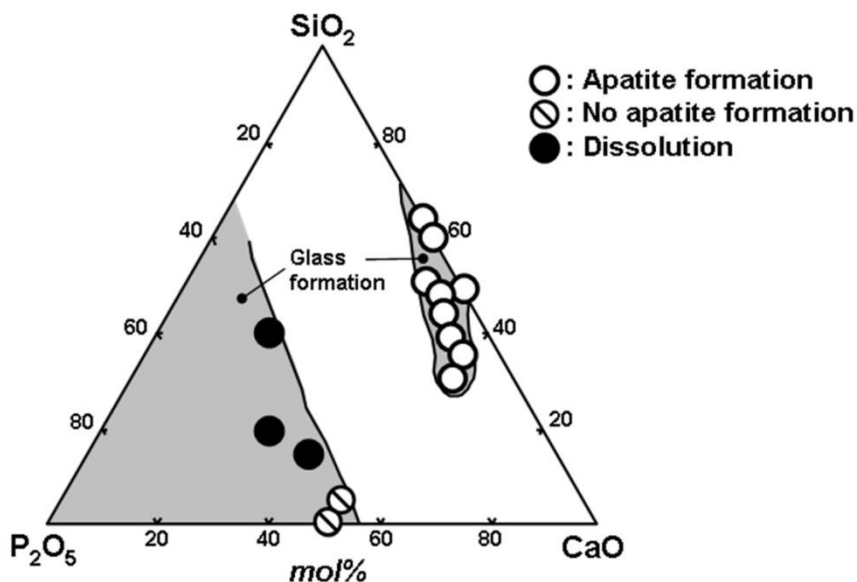
Once an apatite layer is formed on a material in the living body, osteogenic cells preferentially proliferate and differentiate on its surface to promote bone formation. This was confirmed by cell culture experiments [37–39]. As a result, the surrounding bone can come into direct contact with the surface apatite. When this occurs, a tight chemical bond is formed between the apatite in the newly grown bone and the surface apatite [40, 41]. It can be concluded from these results that a material that is able to form an apatite layer on its surface in SBF bonds to living bone through the apatite layer formed on its surface in the living body, as far as it contains neither a cytotoxic nor antigenic component.

Our current interest is to determine the kind of material that forms apatite on its surface in SBF. Ohtsuki et al. [28] showed in a study of the apatite-forming abilities of glasses in the

system $\text{CaO-SiO}_2\text{-P}_2\text{O}_5$ that an apatite layer is formed on CaO , SiO_2 -based glasses but not CaO , P_2O_5 -based glasses in SBF, as shown in Figure 4-3. Both of them increase the ionic activity product of apatite in SBF, which is already higher than the solubility product even before the soaking of the glasses, due to a releasing of calcium or phosphate ions. Despite this, only the CaO , SiO_2 -based glasses form the surface apatite. This is attributed to the catalytic effect for apatite nucleation of functional groups, such as Si-OH , which formed on their surfaces. Once the apatite nuclei are formed, they can grow spontaneously by consuming the calcium and phosphate ions in SBF, since SBF is highly supersaturated with respect to the apatite. This indicates that a material having groups functionally effective for apatite nucleation on its surface forms the apatite.

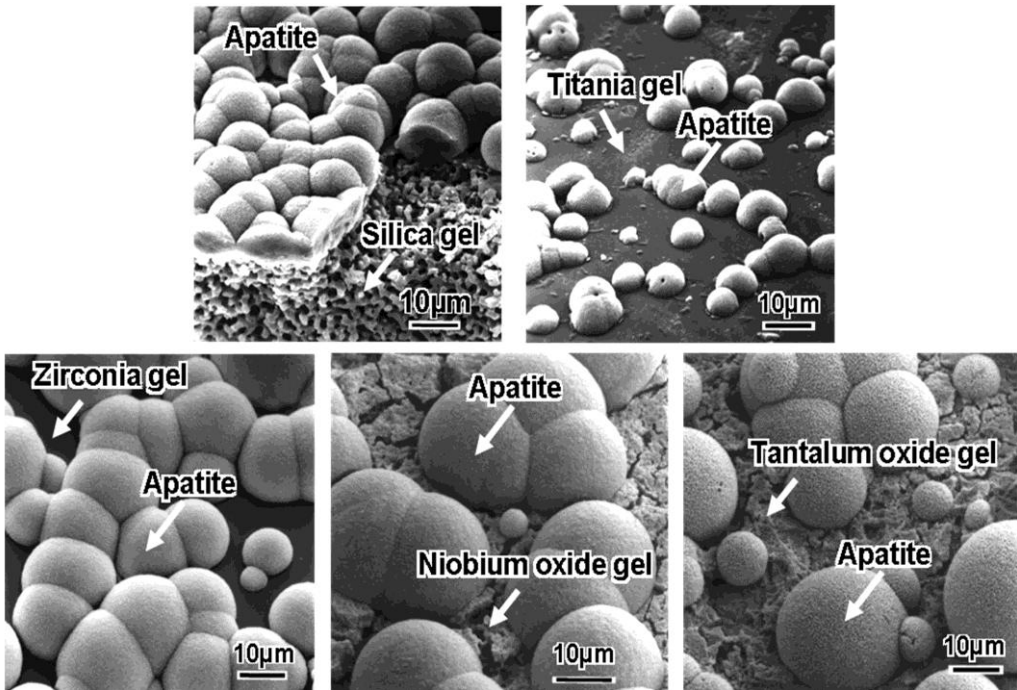
We have an interest in determining the kind of functional group that is effective for apatite nucleation. Pure metal oxide gels of SiO_2 [42], TiO_2 [43], ZrO_2 [44], Nb_2O_5 [45] and Ta_2O_5 [46] were found to induce surface apatite formation in SBF, as shown in Figure 4-4. It was assumed from these results that the Si-OH , Ti-OH , Zr-OH , Nb-OH and Ta-OH groups abundant on their surfaces are effective for apatite nucleation. Later, it was also shown that the COOH [47], PO_4H_2 [47] and SO_3H [48] groups are also effective for apatite nucleation. These functional groups are negatively charged in the physiological body environment.

On the basis of these findings, a bioactive Ti metal and its alloys were developed by forming a sodium titanate on their surfaces through an application of NaOH solution and subsequent heat treatment [49]. Thus, the treated Ti metal and its alloys are negatively charged in the body environment to induce apatite formation on their surfaces, as shown in Figure 4-5 [50-53] and bond to living bone through the apatite layer, as shown in Figure 4-6 [54]. These treatments were applied to the porous Ti metal layer of an artificial hip joint and have clinically been in use in Japan since 2007, as shown in Figure 4-7 [55,56].



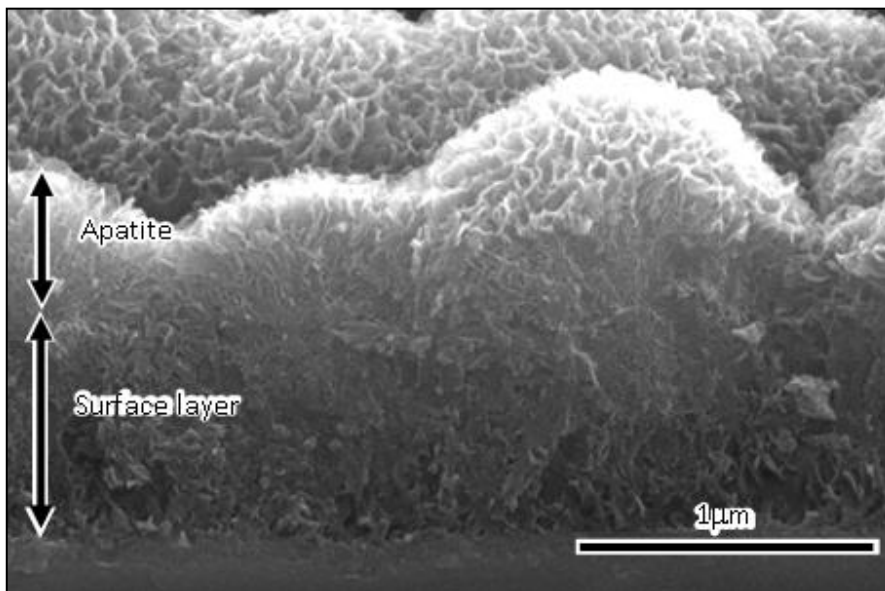
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Figure 4.3. Compositional dependence of apatite formation on the surface of glasses in the $\text{CaO-P}_2\text{O}_5\text{-SiO}_2$ system after soaking in SBF for 28 days. CaO , SiO_2 -based glasses form apatite, whereas CaO , P_2O_5 -based glasses do not form apatite [28].



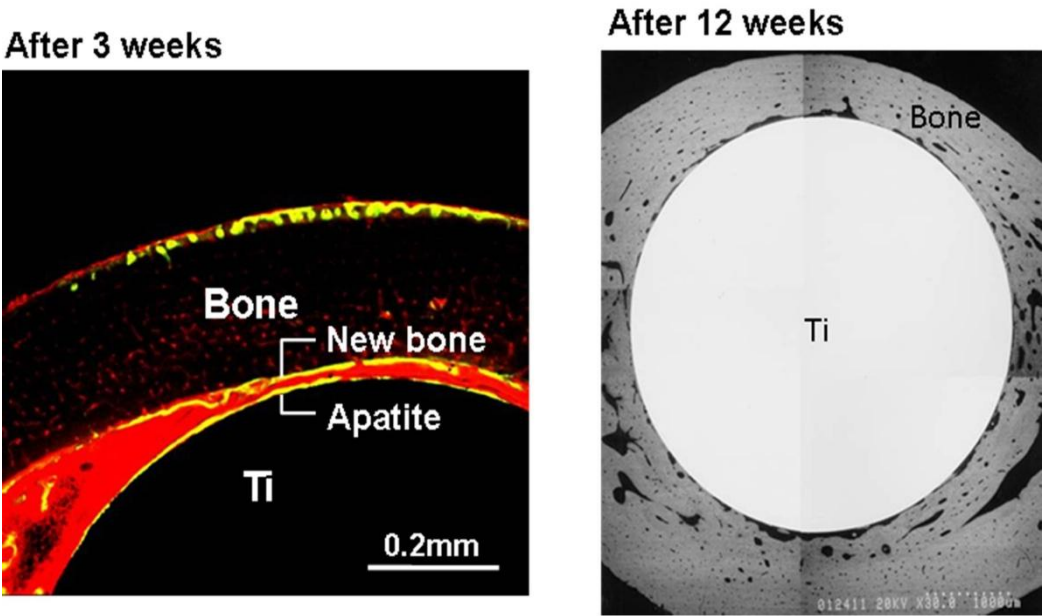
[42-44], Copyright 1992, John Wiley and Sons. [45], Copyright 2001, The Ceramic Society of Japan. [46], Copyright 2001, Journal of Sol-Gel Science and Technology.

Figure 4.4. Apatite formation induced on various metal oxide gels in SBF [42-46].



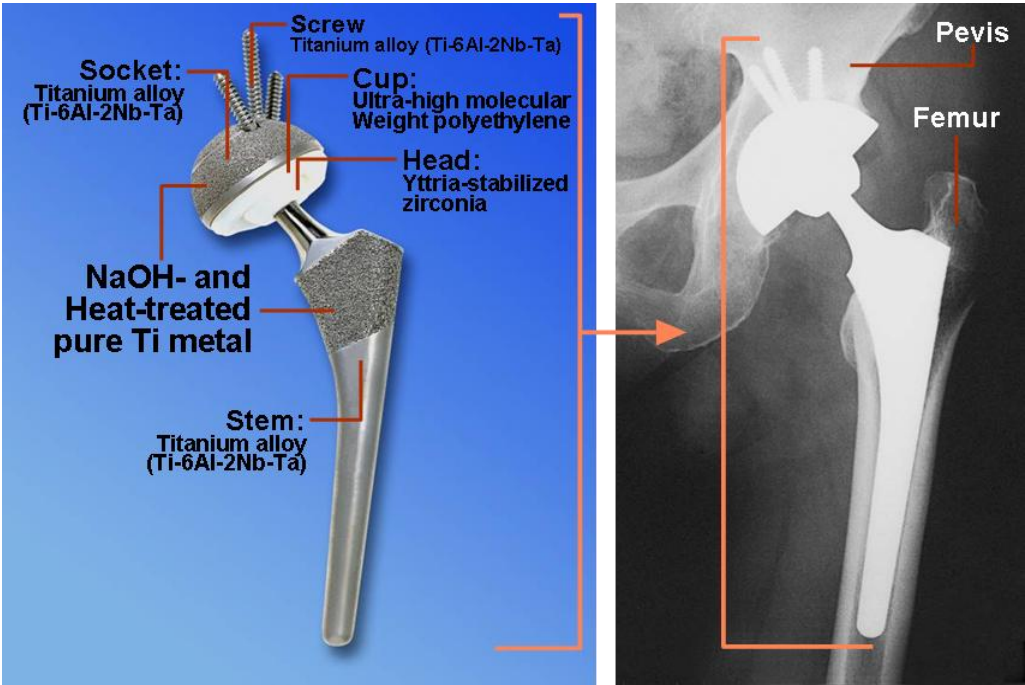
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Figure 4.5. Cross-sectional SEM image of apatite formed on NaOH- and heat-treated Ti metal after soaking in SBF for one day [53].



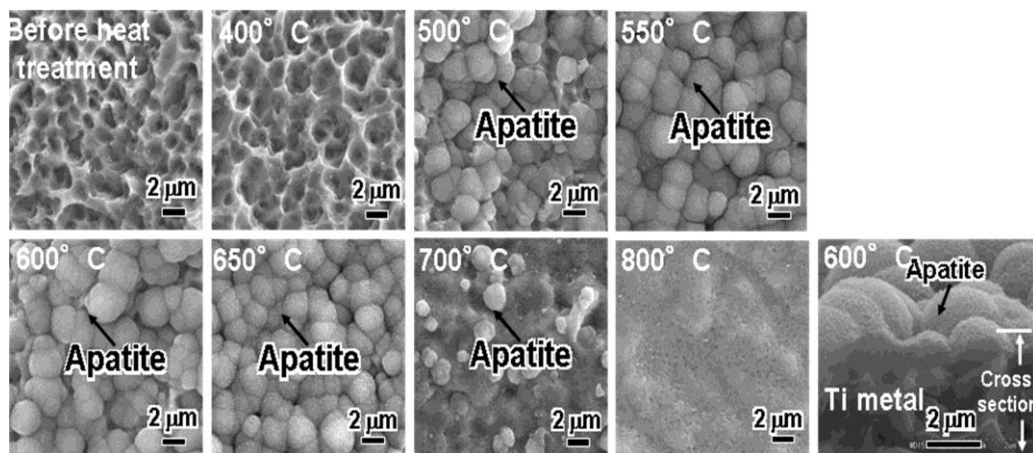
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Figure 4.6. Confocal laser scanning microscope photograph (left) and SEM photograph (right) of the cross-section of the NaOH- and heat-treated Ti metal implanted in rabbit femur for 3 and 12 weeks [54].



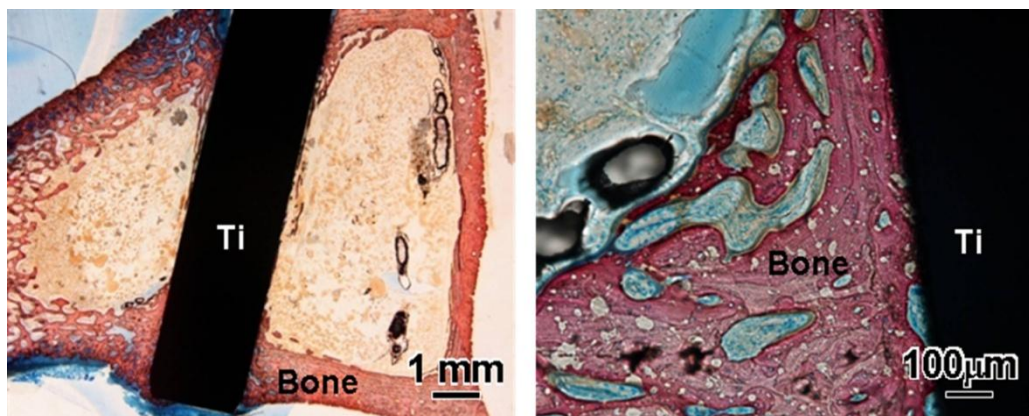
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Figure 4.7. Clinical application of NaOH- and heat-treated Ti metal in hip joint system [55].



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Figure 4.8. SEM photographs of apatite formed on Ti metal soaked in SBF for one day, after heat treatment at various temperatures following $\text{H}_2\text{SO}_4/\text{HCl}$ mixed acid treatment [57].



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Figure 4.9. Optical photographs of strained sections of acid- and heat-treated Ti metal implanted into rabbit tibia for four weeks [57].

Recently, it was found that a bioactive Ti metal is also obtainable through the surface formation of titanium oxide through an application of acid solution and subsequent heat treatment [57]. The treated Ti metal is positively charged in the body environment so as to induce surface apatite formation, as shown in Figure 4–8, and bonds to living bone through the apatite layer, as shown in Figure 4–9.

4.3. The SBF Test Procedure

The following procedure is recommended for the SBF test. This procedure is based on ISO standard 23317 [58] but is slightly revised based on comments given to the standard.

Table 4.1. Nominal ion concentrations of SBF in comparison with human blood plasma

Ion	Concentration (mM=mmol/L)	
	SBF (pH 7.40)	Blood plasma (pH 7.2 to 7.4)
Na ⁺	142.0	142.0
K ⁺	5.0	5.0
Mg ²⁺	1.5	1.5
Ca ²⁺	2.5	2.5
Cl ⁻	147.8	103.0
HCO ₃ ⁻	4.2	27.0
HPO ₄ ²⁻	1.0	1.0
SO ₄ ²⁻	0.5	0.5

4.3.1. Preparation of the Simulated Body Fluid (SBF)

4.3.1.1. SBF Ion Concentrations

The ion concentrations in SBF are shown in Table 4–1 in comparison with the ion concentration in human blood plasma.

4.3.1.2. SBF Reagents

The following reagents are required for preparing SBF. Among them, the powder reagent grade chemicals have to be stocked in a desiccator. Ion-exchanged and distilled water is used for the preparation of SBF:

- (1) sodium chloride (NaCl)
- (2) sodium hydrogen carbonate (NaHCO₃)
- (3) potassium chloride (KCl)
- (4) di-potassium hydrogen phosphate trihydrate (K₂HPO₄·3H₂O)
- (5) magnesium chloride hexahydrate (MgCl₂·6H₂O)
- (6) calcium chloride (CaCl₂) or calcium chloride dihydrate (CaCl₂·2H₂O)
- (7) sodium sulfate (Na₂SO₄)
- (8) Tris-hydroxymethyl aminomethane: ((HOCH₂)₃CNH₂) (TRIS)
- (9) 1M (mol/l) Hydrochloric Acid, 1M-HCl
- (10)pH standard solution, (pH 4, 7 and 9)

4.3.1.3. Preparation of SBF

Since SBF is supersaturated with respect to apatite, an inappropriate preparation method can lead to the homogeneous precipitation of apatite in the solution. During preparation, the solution should remain colourless, transparent and without deposits on the surface of the bottle. If any precipitation occurs, stop preparing the SBF, abandon the solution and restart by washing the apparatus. In Table 4–2, the reagents for the preparation of 1000mL of SBF are given in the required order of dissolution.

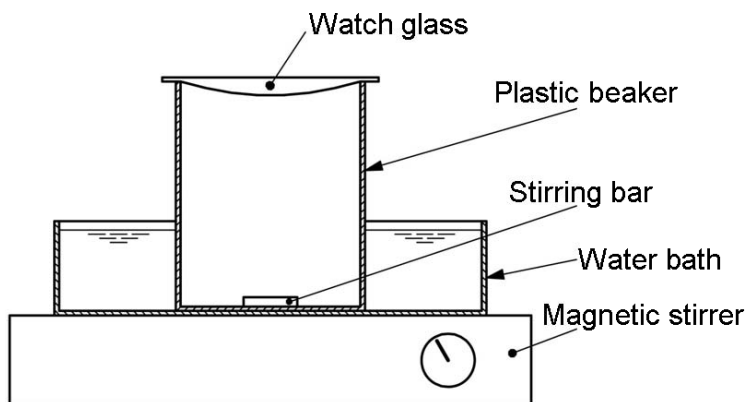
Table 4.2. Order and an example of the amounts of reagents for preparing 1000mL of SBF

Order	Reagent	Amount ^a	Container	Purity ^b	Formula weight
1	NaCl	8.035 g	weighing paper	99.5 %	58.4430
2	NaHCO ₃	0.355 g	weighing paper	99.5 %	84.0068
3	KCl	0.225 g	weighing bottle	99.5 %	74.5515
4	K ₂ HPO ₄ ·3H ₂ O	0.231 g	weighing bottle	99.0 %	228.2220
5	MgCl ₂ ·6H ₂ O	0.311 g	weighing bottle	98.0 %	203.3034
6	c(HCl) = 1 mol/L	39 mL	graduated cylinder	—	—
7	CaCl ₂ ^c	0.292 g	weighing bottle	95.0 %	110.9848
8	Na ₂ SO ₄	0.072 g	weighing bottle	99.0 %	142.0428
9	TRIS	6.118 g	weighing paper	99.0 %	121.1356
10	c(HCl) = 1 mol/L	0 to 5 mL	syringe dropper	—	—

^a The amounts of the reagents are changed depending upon their purities.

^b The purity given in this table is typical of one reagent available in most countries.

^c If calcium chloride dihydrate CaCl₂·2H₂O is used, attention shall be given to the different molar weight; amount of reagent: 0.371 g, Purity of reagent: 99.0 %, formula weight 147.0152.



Copyright 2007. ISO.

Figure 4.10. Example of apparatus for preparing SBF [58].

In order to prepare 1000mL of SBF, first put 700 mL of ion-exchanged and distilled water, with a stirring bar, into a 1000mL plastic beaker. Set it in the water bath on the magnetic stirrer, and cover it with a watch glass or plastic wrap. Heat the water in the beaker to $36.5\text{ }^{\circ}\text{C} \pm 1.5\text{ }^{\circ}\text{C}$ whilst stirring. Figure 4–10 shows an example of the apparatus used for preparing SBF.

Dissolve the first to eighth reagents in the order given in Table 4–2 in the distilled water at $36.5\text{ }^{\circ}\text{C} \pm 1.5\text{ }^{\circ}\text{C}$, keeping the following in mind:

- Glass containers should be avoided. A plastic container, with a smooth surface and without any scratches, is recommended, because apatite nucleation can be induced at the surface of a glass container or the edges of scratches.

- b) Dissolve a reagent only after the preceding one (if any) is completely dissolved.
- c) The reagent $\text{CaCl}_2/\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ has a great effect on the precipitation of apatite. Therefore, dissolve the $\text{CaCl}_2/\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ little by little.
- d) Rinse the graduated cylinder with 1 mol/L-HCl before measuring the volume of 1 mol/L-HCl.
- e) Measure the hygroscopic reagents such as KCl, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CaCl}_2/\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Na_2SO_4 as quickly as possible.

Insert the electrode of the pH meter into the solution. Just before dissolving the TRIS, the pH of the solution should be 2.0 ± 1.0 . Set the temperature of the solution at $36.5^\circ\text{C} \pm 1.5^\circ\text{C}$. If the amount of the solution is smaller than 900mL, add distilled water in an amount up to 900 mL in total.

With the solution temperature between 35°C and 38°C , preferably $36.5^\circ\text{C} \pm 0.5^\circ\text{C}$, dissolve TRIS into the solution little by little, taking careful note of the pH change. During the SBF preparation, bubbles are likely to attach to the electrode surface of the pH meter and prevent an accurate measurement of the pH. The electrode should be shaken gently every five to ten minutes to remove the bubbles. After adding a small amount of TRIS, wait until the reagent is dissolved completely and the pH has become constant. Then add another small amount of TRIS.

It is recommended not to add a large amount of TRIS into the solution at once, because a radical increase in the local pH of the solution could lead to the precipitation of apatite. The following procedure is recommended: If the solution temperature is not within $36.5^\circ\text{C} \pm 0.5^\circ\text{C}$, add TRIS to raise the pH to 7.3 ± 0.05 , then stop adding and wait for the solution temperature to reach $36.5^\circ\text{C} \pm 0.5^\circ\text{C}$. With the solution at $36.5^\circ\text{C} \pm 0.5^\circ\text{C}$, add more TRIS to raise the pH to a point under 7.45. The pH should not increase to over 7.45 at $36.5^\circ\text{C} \pm 0.5^\circ\text{C}$, taking account of the pH decrease with the increasing solution temperature.

Make sure that the temperature of the solution is maintained at $36.5^\circ\text{C} \pm 0.5^\circ\text{C}$. When the pH has risen to 7.45 ± 0.01 , stop dissolving the TRIS, then add HCl solution by syringe to lower the pH to 7.42 ± 0.01 , taking care that the pH does not decrease below 7.40. After the pH has fallen to 7.42 ± 0.01 , dissolve the remaining TRIS little by little until the pH has risen to ≤ 7.45 . If any TRIS remains, add the 1 mol/L -HCl and TRIS alternately into the solution. Repeat this process until the whole amount of TRIS is dissolved, keeping the pH within the range of 7.42 to 7.45. After dissolving the whole amount of TRIS, adjust the temperature of the solution to $36.5^\circ\text{C} \pm 0.2^\circ\text{C}$. Adjust the pH of the solution by adding HCl solution little by little at a pH of 7.42 ± 0.01 at $36.5^\circ\text{C} \pm 0.2^\circ\text{C}$ and then finally adjust it to 7.40 exactly at 36.5°C on the condition that the rate of solution temperature increase or decrease is less than $0.1^\circ\text{C}/\text{min}$. Remove the electrode of the pH meter from the solution, rinse it with distilled water and add the washings to the solution.

Pour the pH-adjusted solution from the beaker into a 1000mL volumetric flask. Rinse the surface of the beaker with distilled water several times and add the rinse to the flask. Fix the stirring bar with a magnet to prevent it from falling into the volumetric flask.

Add the distilled water up to the marked line (it is not necessary to adjust this exactly, because the volume is reduced after cooling). Put a lid on the flask, and close it using a plastic film. After mixing the solution in the flask, keep it in the water bath so that it cools to 20°C . After the solution temperature has fallen to 20°C , add distilled water up to the marked line.

4.3.1.4. Confirmation of the Ion Concentration of SBF

The prepared SBF should have the ion concentrations shown in Table 4–1. In order to confirm the ion concentrations of the SBF, chemical analysis of the SBF is recommended because SBF is a metastable solution supersaturated with respect to apatite. It is also recommended that the apatite-forming ability of the standard glasses in the prepared SBF be examined. Chemical compositions of the standard glasses for evaluating the apatite-forming ability are shown in Table 4–3. When the standard glasses A, B and C for evaluating apatite-forming ability are soaked in SBF, an apatite layer should be detected by a thin film X-ray diffraction (TF-XRD) pattern after soaking for 12 h, 24 h and 120 h.

Table 4.3. The compositions of the standard glasses in the Na₂O–CaO–SiO₂ system

Standard glass	Composition /mol%		
	SiO ₂	Na ₂ O	CaO
Glass A	50	25	25
Glass B	55	22.5	22.5
Glass C	60	20	20

4.3.1.5. Preservation of SBF

Prepared SBF should be preserved in a plastic bottle with a lid put on tightly and kept at $7.5^{\circ}\text{C} \pm 2.5^{\circ}\text{C}$ in a refrigerator. The SBF should be used within 30 days of preparation.

It is recommended that the final product of SBF be sterilized by filtration to eliminate any dust particles and bacteria from the fluid, since dust can promote the heterogeneous nucleation of apatite, and bacteria often phagocytoses the apatite formed in SBF. The sterilization should be carried out just after the preparation and/or just before the evaluation of the apatite-forming ability. For sterilization just after preparation (before preservation), the whole amount of as-prepared SBF is filtered using a peristaltic pump through a sterile vented filter unit with a pore size of 0.22 μm at the flow rate of 85–100 mL/min. For sterilization just before starting evaluation for apatite-forming ability, the SBF is filtered by using a syringe attached with a sterile vented filter unit with a pore size of 0.22 μm . Either or both of these sterilization methods may be used.

4.3.2. Test of Apatite Formation on Sample

For dense materials, measure the specimen dimensions to an accuracy of ± 0.1 mm and calculate the surface area to an accuracy of 2 mm² for a thin plate.

Calculate the volume of SBF that is used for testing using the following equation:

$$V_s = 100 S_a$$

where

V_s is the volume of the SBF (mm³); S_a is the apparent surface area of the specimen (mm²).

For porous materials, the volume of SBF should be greater than the calculated Vs. Put the calculated volume of SBF into a plastic bottle with a cap.

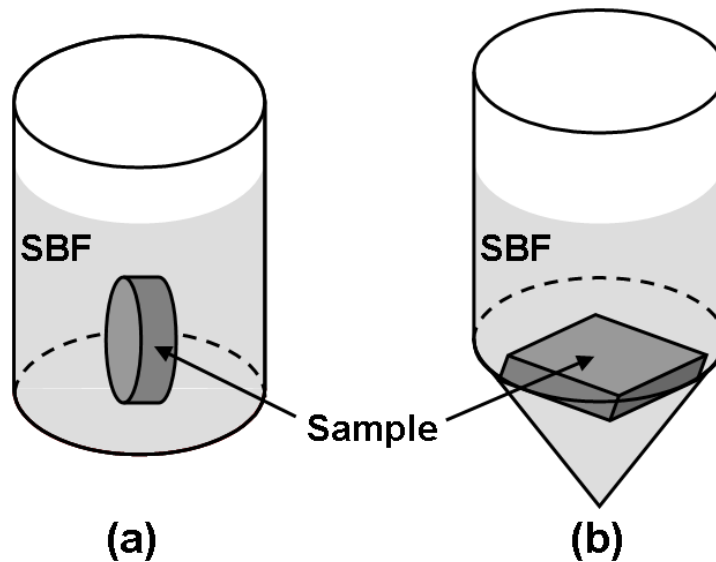


Figure 4.11. Arrangements of specimen in the SBF.

After heating the SBF to 36.5 °C, a specimen is placed in the SBF, as shown in Figure 4–11. The entire specimen should be submerged in the SBF. In rare cases, apatite can homogeneously precipitate in the SBF and become deposited on the surface of a specimen. Therefore, it is recommended that the specimens be placed in the SBF as shown in Figure 4–11 a) or Figure 4–11 b). In case of the placement as shown in Figure 4–11 b), apatite formation should be examined on the lower surface of the specimen.

After soaking in the SBF at 36.5 °C for different periods, take out the specimen from the SBF and gently rinse it with distilled water. A soaking time for test is recommended to be set less than four weeks.

The specimen is then dried in a desiccator without heating. A specimen, once taken out of SBF and dried, is not to be soaked again.

Bone bonding materials usually form apatite on their surfaces within four weeks.

Examine the surface of a specimen by TF-XRD and/or SEM to determine whether apatite is detected. It is recommended to perform the TF-XRD measurement in the range of 3° to 50° in 2 theta (Θ) using $\text{CuK}\alpha$ ($\lambda = 0,154\ 05\ \text{nm}$) radiation as the source at a rate of 20/min and a 1° glancing angle against the incident beam on the specimen surface.

The dried specimen for SEM observation should be thinly coated with metal to induce electro conductivity. The SEM photos should be taken at both high magnification (approximately X 10,000) and low magnification (approximately X 1,000).

The TF-XRD measurement can clearly identify the apatite formation on the specimen. The SEM observation can determine material formation on the specimen but cannot identify whether apatite is formed or not. Therefore, the SEM observation should be accompanied with the TF-XRD measurement. However, formed apatite grains and layers have

characteristic features to be identified, and the apatite formation is sometimes estimated only on SEM.

Conclusion

Materials that form a surface apatite layer on their surfaces in the living body bond to living bone through the apatite layer insofar as they contain neither cytotoxic nor antigenic components. Certain resorbable materials are replaced with living bone after forming the apatite on their surfaces or bond to living bone without forming detectable apatite layer on their surfaces.

Apatite formation on synthetic materials *in vivo* can be reproduced *in vitro* in acellular simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma. Therefore, the bone-bonding ability of a material may be roughly determined by examining its apatite-forming ability in SBF. If the SBF test is used carefully, it is useful for selecting a bone-bonding material prior to animal experiments. By using the SBF test, the cost and term of animal experiments, as well as the number of animals required for sacrifice, can be markedly reduced.

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Chapter V

Using *In Vitro* Cell Culture Models to Evaluate Implant Surfaces

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Abstract

Osseointegration is a well-described clinical phenomenon with very high success rates. However, the development of the bone-implant interface occurs by a process of cellular intervention that involves the development of a blood clot with platelets activation, interaction of monocytes from the clot and early recruitment and adhesion of mesenchymal stem cells and osteoprogenitors. The maintenance of this interface, while less well described at the cellular and molecular level is attributed to osteoblast / osteoclast interactions that reflect the biological and mechanical environment of the endosseous implant. Additional knowledge has been gained by the adoption of improved cell culture models of osteogenesis and osteoinduction, through the additional consideration of other cell types involved in osseointegration, and by more detailed analysis of cell behavior as a function of a modified implant surface. The aim of this chapter is to consider what cell culture models have been used to investigate the process of osseointegration, get more insight on how this process occur at the cellular/molecular level and to discuss what has been learned about osseointegration by using the different cell culture models.

5.1. Introduction

Osseointegration is a well-described clinical phenomenon. The fixation of an alloplastic root analog in alveolar bone is associated with the histological representation of a direct apposition of bone with the endosseous implant surface. The quality of the tissue relationship

with the alloplastic surface requires a minimal bone-to-implant contact and maintenance of vital bone volume during the functional lifetime of the implant. The development of the bone-implant interface occurs by a process of cellular intervention that involves the development of a blood clot with adherent, activated platelets, the interaction of monocytes from the clot and early recruitment and adhesion of mesenchymal stem cells and osteoprogenitors. The maintenance of this interface, while less well described at the cellular and molecular level is attributed to osteoblast / osteoclast interactions that reflect the regional biologic and mechanical environment of the endosseous implant. Learning precisely how tissue responses to alloplastic materials are mediated at a cellular level involves interrogation using multiple approaches. Included are clinical, radiographic, histologic, cellular and molecular methods. The aim of this review is to consider what cell culture models have been used to investigate the process of osseointegration and to discuss what has been learned.

Different cell types and models have been used to evaluate osseointegration process (Table 5–1). This was addressed in a comprehensive review over a decade ago. [1-3] Additional knowledge has been gained by the adoption of improved cell culture models of osteogenesis and osteoinduction, through the additional consideration of other cell types involved in osseointegration, and by more detailed analysis of cell behavior as a function of a modeled alloplastic interfacial surface.

5.1.1 Modeling the Osteoblast Role in Osseointegration

Cell culture models of osseointegration involve the growth of cells adherent to a substrate representing the endosseous implant surface (e.g., Titanium, Hydroxyapatite, Zirconia). In all cases, these models support cellular growth using defined culture media supplemented with serum to provide growth factors and plasma proteins (Figure 5–1). In addition, osteoinduction is supported by the use of well-defined supplements that have been shown in fundamental models of osteoinduction and osteogenesis to direct cellular differentiation along the osteoblast pathway [72,73].

The differentiation of implant substrate-adherent progenitors requires the use of such supplements. Only in rare cases has a modeled alloplastic substrate induced the osteoblastic differentiation of adherent cells without such osteogenic supplements. [74] The great majority of cell culture studies performed on model endosseous implant substrates have employed osteogenic supplements and, thus, have investigated the role of surface as a mediator of the induced differentiation process. This may reflect the *in vivo* scenario in which the osteotomy or wounding of bone and bone marrow incite the osteoinductive program that is modulated by cell-surface interactions.

Different cell types have been used as models for osteoblast differentiation and bone formation *in vitro* (Table 5–2). These culture systems have been categorized as primary cultures, non-transformed cell lines, osteosarcoma cell lines, and conditionally immortalized cell lines. This classification reflects the origin of cell lines, but not the features of each model.

Examples of primary cultures are: fetal rat calvarial model, [75,76] rat bone marrow stromal cells, [62,77-80] fetal bovine osteoblasts, [81] human embryonic [82] and adult osteoblasts, [83,84] human gingival fibroblasts, [85-87] and human mesenchymal stem cells (hMSCs). [8,9,72,88-90].

Table 5.1. Cell Culture Models of Osteoblast Response at Different Implant Surfaces

Cell culture model	Cell response	Ref.
Human mesenchymal stem cells - hMSCs	Changes in cell cytoskeleton	[4,5]
	Cell Differentiation	[6-9]
Primary human osteoprogenitors	Cell Differentiation	[10]
Human Bone marrow cells	Cell Filopodia	[11]
	Cell Proliferation, Differentiation, OPG expression, Ca deposition	[12]
Human fetal osteoblasts - hFOB	Cell Adhesion	[13]
	Cell Signaling	[14]
	Cell Adhesion – Differentiation	[15]
Human bone-derived osteoblasts	Cell Signaling	[16]
	Cell Adhesion - Changes in cell cytoskeleton	[17]
	Cell Adhesion	[18-21]
	Cell Adhesion – Ca deposition	[22]
	Cell Adhesion – Differentiation – Ca deposition	[23,24]
	Cell Proliferation	[25]
	Cell Proliferation – Differentiation	[26]
	Cell Differentiation – Ca deposition	[27]
	Apoptosis	[28]
	Osteoblast Specificity	[29-31]
Primary murine bone marrow (mMSCs)	Cell Adhesion – Proliferation - Differentiation – Ca deposition	[32]
	Cell Adhesion	[33]
Osteoblast – MC3T3-E1	Cell Adhesion	[34-37]
	Cell proliferation	[38]
	Cell Adhesion – Differentiation– Ca deposition	[39-43]
Osteoblast – neonatal rat calvaria	Changes in cell cytoskeleton	[44]
	Cell Adhesion	[45-49]
	Cell Differentiation	[50-53]
	Cell Differentiation – Ca deposition	[49,54-58]
	Osteoblast Specificity	[59]
Rat bone marrow cells	Cell Adhesion – Proliferation - Differentiation	[60-62]
	Cell Differentiation	[63]
	Cell tensile test resistance	[64,65]
Osteoblast-like MG63	Cell Proliferation	[66]
	Cell Differentiation	[67-71]
Saos-2 cell line	Cell Differentiation	[71]

Mesenchymal stem cells or synonymously marrow stromal cells isolated from the bone marrow are advantageous because they are multipotent undifferentiated cells that can differentiate along several different pathways, including osteogenic, chondrogenic and adipogenic lineages, depending on culture conditions. [91,92].

Table 5.2. Cell Culture Models of Osseointegration

Model	Advantages	Disadvantages
Primary culture (e.g., bone marrow derived MSC, calvaria)	• Can represent entire differentiation stages • Form a rich bone matrix in culture	• Limited passage number
Cell culture osteo-progenitors (e.g - MC3T3E1, C3H10T1/2)	• Stability through many passages, • Differentiated using media supplements	• Cells may represent different stage of differentiation along differentiation lineage
Transformed cell lines (e.g. FOB, MG-63)	• Stability, immortal, • Differentiated using media supplements	• Transformation may alter phenotype;

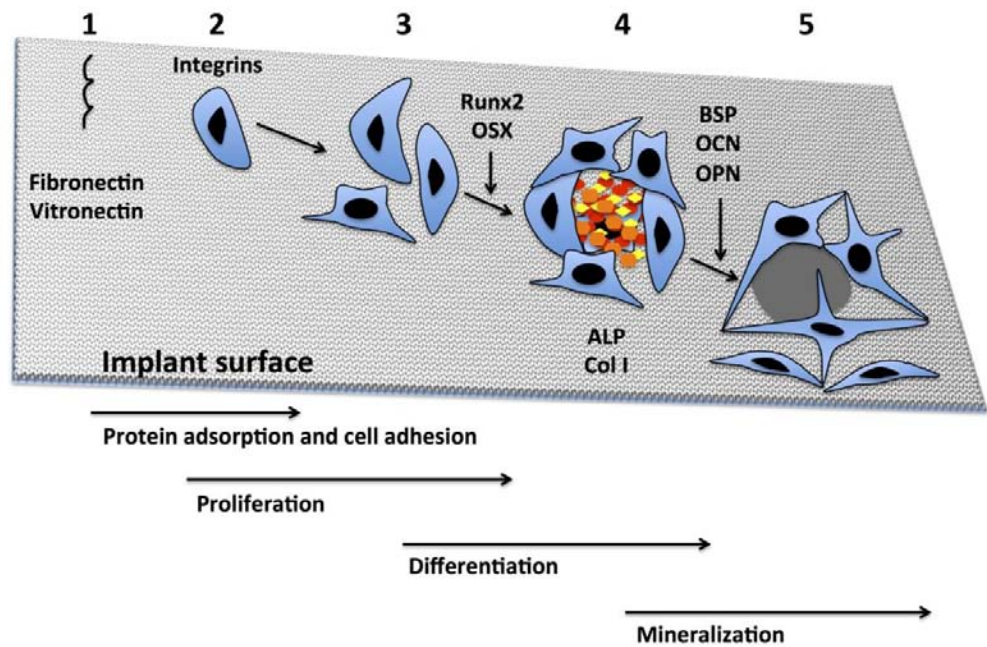


Figure 5.1. Temporal pattern for the formation of bone matrix at the implant surface. A layer of proteins (of special interest are the ones that contains RGD domains – Fibronectin and vitronectin) will adsorb on the surface and recruited stem cells will adhere to the surface via their integrin receptors. Initial differentiation is induced with osteogenic supplements added to the cell culture medium. Transcriptions factors (Runx2 and OSX) are produced by the cells and induce osteoblast differentiation. Collagen (Col I) and alkaline phosphatase (ALP) are expressed earlier (early osteogenic differentiation markers), while bone sialoprotein (BSP) and osteocalcin (OCN) are expressed later and precedes matrix mineralization. Osteopontin (OPN) is expressed later in culture and is coincident with mineral accumulation at the implant-bone interface. This pattern of gene expression is observed at implant surfaces and is modulated by different characteristics of each surface.

However, experiments using primary cell types does not always show reproducible results, due to the biological variation between samples used in each experimental run. [80,93] Moreover, primary cultures derived from normal human bone have an osteoblastic

phenotype but proliferate at a very slow rate and become senescent after a relatively short time in culture. [94].

The most common examples of a non-transformed cell line are the clonal MC3T3-E1 cell line derived from mouse calvaria [95] and C3H10T1/2 cells. [96,97] MC3T3-E1 cell line is considered a committed osteoprogenitor and may not recapitulate the earliest aspects of osteoinduction. However, MC3T3-E1 cells permit analysis of many important osteogenic signaling pathways. [42] The mouse calvaria C3H10T1/2 cells display fibroblastic morphology, and are also able to develop into osteoblasts, chondrocytes, as well as adipocytes under specific inductions. [97].

Some examples of transformed cells derived from osteosarcoma are: human MG63 osteoblast-like cells, [67,98-100] Saos-2, [101-103] U2OS, [104] and rat osteosarcoma-derived cell lines UMR-106, [105] and ROS 17/2.8. [106] MG63 cells, despite being a tumor cell line, which proliferates more rapidly than human bone derived cells, exhibit many osteoblastic traits which are characteristic for bone forming cells. [98] According to Rodan et al., [101] human Saos-2 cells share many properties with the rat osteosarcoma ROS 17/2.8 cell line.

Conditionally immortalized cell lines include human fetal osteoblasts (hFOB)s, [94,107,108] and HOBIT (Human osteoblast-like initial transfectant) cell line. [109] hFOB)s are conditionally immortalized with a gene coding for a temperature-sensitive mutant (tsA58) of SV40 large T antigen, [94] while HOBIT cell line is derived from primary cultures of human bone cells transfected with a gene expressing the SV40 large T antigen. [109] hFOB)s and HOBIT, as clonal cell lines, have much less variability and studies using these cells lines should be highly reproducible. [94,109].

Maniatiopoulus et al. [78] affirmed that cells derived from osteosarcoma cannot totally differentiate *in vitro*, while immortalized lineage can present different phenotypic expression of the cells from which they originated. Also, an immortalized cell line is relatively easier to maintain with many passages over a long period of time without a loss of phenotypic expression. [93] The disadvantages of using nontransformed cell lines is that as they are isolated from different donors they show a large variability in differentiation, attachment and integrin expression, which severely complicates the evaluation of obtained results. Although these variations are not encountered in transformed cell lines, which are a more homogeneous cell population, they have lost many of their osteogenic features. [104] Transformed cell lines show an inverse correlation between cell proliferation and differentiation. [110].

5.1.2. Modeling Initial Cellular Responses at Endosseous Implants in Cell Culture

Although the goals of those *in vitro* (cell culture) studies often extend to drawing broader conclusions about osseointegration, they fall short in this respect because only specific cell types are investigated and these investigations have been largely limited to the osteoblast lineage. The main disadvantage of cell culture models is the limitation in mimicking the *in vivo* environment that fundamentally involves many more cell types. A dental implant is placed into bone marrow; it is surrounded by hemorrhage and a resulting blood clot, and incites a wound-healing cascade that involves cells of the immune system. An osteoblast-

specific focus in cell culture likely offers an incomplete assessment of the process of osseointegration.

A recent molecular assessment of the osteogenic responses of cells adherent to implants retrieved from human alveolar bone at 24 and 72 hours revealed little or no evidence of osteoblastic precursors or osteoinduction. [111] One interpretation is that early populations of implant adherent cells include other cell types. In fact, additional assessments of inflammatory cell markers confirm that immune cells including monocytes and T-cells may contribute to the early population of implant adherent cells. This is consistent with general knowledge of wound healing and the role of inflammatory cells. Earlier investigations have also highlighted the importance of the blood clot and contact osteogenesis. [112,113] Additionally, the interaction of platelets with implant surfaces may influence the process of osseointegration. These *in vivo* observations merit consideration of the body's earliest cellular responses to implanted alloplastic surfaces.

Toward this end, several investigators have pursued defining the role of surface variation in chemistry and topography on the behavior of cells other than osteoblasts. [114-118] Platelet degranulation, for example, is apparently enhanced by increasing surface topography and has been interpreted to represent positive influence on subsequent healing events. [119] Several different laboratories have utilized similar models of platelet adhesion and degranulation to demonstrate that the nature of the implant surface may potentially influence this key step in the wound healing process.

The initial interaction of implant surfaces in the body likely involves the adherence of serum proteins and circulating blood cells. Primary among these cells are blood platelets that have central roles in wound healing. [120] Initial cell culture studies supported the logical observation that platelets interact directly with the implant surface and are integral to the formed fibrin blood clot. Several investigations have modeled this interaction using human platelets and implant surfaces in the laboratory. Steinberg et al [121] demonstrated by *in vivo* insertion of hydroxyapatite and titanium implants that within 120 seconds of insertion, implant surfaces promoted initial clot formation. In contrast, the authors suggested that tooth roots with intact periodontal ligament supported complete clot formation within this brief period. This interaction is dependent upon the CpIIb/IIIa integrin receptors on platelets [122] and adsorbed serum proteins. The titanium oxide surface supports immediate (5 seconds) platelet adhesion by preceding adhesion of serum proteins. [123,124] Surface roughness altered the initial blood cell/implant interactions.[125] Greater numbers of platelets adhered from blood and greater platelet activation on to a dual acid etched surface than a machined surface and that these earliest interactions underscore the process of osteoconduction. [126].

Subsequent studies indicated that surface microtopography and not chemistry (e.g., CaPO_4) is responsible for enhanced platelet attachment and activation. [127] The concept of 'contact osteogenesis' invokes a predominant role for the physical interaction of the blood clot in osseointegration. Additional biochemical roles for the included platelets include the release of PDGF, TGF- β and VEGF prominent among other growth factors and cytokines. It is suggested this contributed to reductions in the time for implant healing. [128] Thor et al [129] indicated that surface modification involving superimposition of nanofeatures (HF-modified TiO_2 grit-blasted) led to increased thrombogenic properties of the surface. Rough surfaces, compared to machined surface appear to increase platelet activation. In a recent evaluation, platelet activation and cytokine release was modeled as a function of titanium surface roughness. Kammerer et al [119] revealed that within 15 – 20 minutes *in vitro*, a

rough SLA (large grit–sandblasted, acid etched) surface supported high platelet consumption and high levels of VEGF and PDGF release. These studies of platelet activation and release should be further investigated in the context of subsequent cellular responses and be modeled in parallel in vivo studies.

The monocyte and macrophage have well defined roles in wound healing. [130] They are particularly implicated in foreign body responses and in particular in terms of particulate debris associated with orthopedic endosseous implants. [131] However, less is known about the adhesion-specific relationship of the monocyte and macrophage in osseointegration. Champagne et al. [117] demonstrated that monocyte/macrophage cell lines displayed adhesion and spreading to titanium in a topography specific manner. Initial studies suggested that these cell lines produced BMP-2 and could influence osteoblastic differentiation in a surface roughness-dependent manner. [117].

Additional studies [132-135] described the topography dependent nature of adherent monocytes. Most recently Hamlet and Ivanovski revealed down regulation of proinflammatory cytokines in cells adherent to nanoscale crystalline CaP-modified titanium alloy. [136] It has also been proposed that osseointegration is a function of the implant–resident cell population and resultant osteoinduction rather than osteoblastic adhesion to an endosseous implant surface alone. [3].

The application and validity of each cell culture system depends on the question to be addressed. Analyses of cell behavior in contact with biomaterial have long been used to investigate the effects of these materials on their biologic environment. Implant surface features; surface, [67,99] chemistry [107,137,138] and energy [139,140] were all found capable of modulating cell differentiation and local factor production. In vitro studies using bone cell cultures provide an efficient screening method and scientific tool in the understanding of the surface biology of implant materials. [1].

However, these settings do not completely resemble the in vivo situation. Therefore, in vivo studies using animal models have been used to provide a higher level of scientific evidence.

5.2. Cell Culture in Implant-Related Research

Cell culture models have been used to study implant-tissue reactions including cytotoxicity and cytocompatibility; protein adsorption and cell adhesion; cell proliferation, differentiation and protein biosynthesis associated with osteogenesis. Different methods have been applied to evaluate the effects of many alloplastic features on all of the biologic issues indicated above and to gain further information about the bone-implant interface (Figure 5–2). Depending on the biological process under investigation, one or a different cell culture model may be utilized (Table 5–2).

Specific considerations may involve selection of species or different cell lines that best represent the biologic problem being pursued. Examples include the repertoire of integrins expressed or the possibility that one or another signaling pathway is altered by the transformation process. Confirmation of in vitro studies in animal models or using multiple cell culture systems should be pursued.

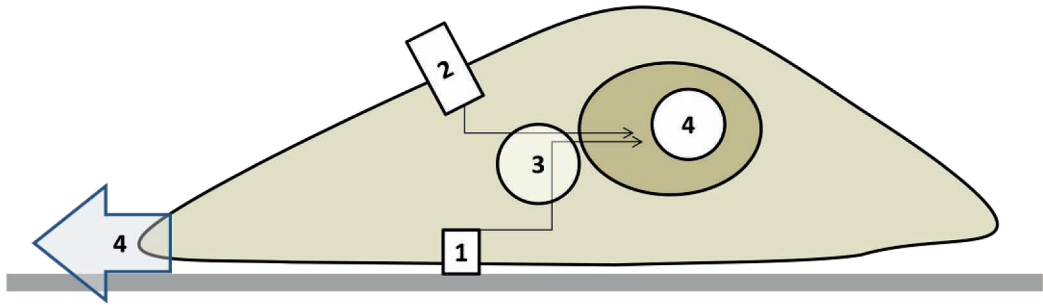


Figure 5.2. The implant-bone interface; Mechanisms modeled in cell culture. An adherent cell responds to the nature of the substrate (dental implant surface). Physical adhesion occurs by specific receptors (1) that provide adhesion but also transduces specific signals to the nucleus to change the phenotype. The cell culture media provide chemical cues for differentiation through additional receptors (2) that transduce added signals (3) to the nucleus. Numerous signaling pathways contribute to this general process illustrated here. Cells that adhere to the surface are capable of moving and motility (4) is also influenced by the nature of the substrate.

5.2.1. Cytotoxicity and Cytocompatibility

In vitro studies are important methods to assess the cytotoxicity of different implant materials as a result of manufacturing and/or sterilization procedures, bulk material, and topographic modifications. Cellular viability assays and the quantification of initial adherent and subsequent number of cells are methods applied to assess cytotoxicity in implant surfaces. Cells may be used to investigate cytocompatibility in two ways: basal cytocompatibility, which relates to common functions (attachment, viability, proliferation, protein synthesis), and specific cytocompatibility, which relates to lineage specific function of the cell (e.g., bone-specific gene expression or mineralization). [26] The basis for cytocompatibility toward one or another material may involve central mediators of inflammation and cellular apoptosis (e.g., NF kappa B) [141] that are affected by the substrate bulk material.

Studies have also demonstrated that the use of osteoblasts instead of fibroblast cultures is supported by observations that osteoblasts and fibroblasts respond differently to culture conditions. [49] Osteoblasts may be more sensitive than fibroblasts to some surface modifications. [26,49,142] Cytocompatibility of materials related to endosseous implants has been considered in terms of related particulate debris and ion release. Debris and ions can affect cell viability and physiology. For example, titanium, aluminum and hydroxyapatite surfaces are cytocompatible, but titanium or hydroxyapatite particles of defined size (which may be present at the implant-bone interface following surgery) [28,143] cause cell death when applied to osteoblast cultures. Also cytotoxicity tests have demonstrated the importance of the size of these particles in osteoblast viability. [28,144,145].

5.2.2. Protein Adsorption and Cell Adhesion

Many of the in vitro studies that attempt to understand implant surface–bone interactions start with cell adhesion. Cell adhesion is an important phenomenon in cell biology, [146] and

the process of attachment is well defined in terms of adhesive extracellular matrix proteins (the biologic substrates for attachment) and a family of trans-membrane cellular receptors—termed integrins [147] - that mediate cell attachment to this substrate. Cell binding to protein domains of adhesive ECM proteins via the integrin receptors transmit signals through collection of proteins on the cytoplasmic face of the contact, termed focal contacts. [148] Cell activity is modulated by the surface roughness as a direct effect of protein interactions at implant surfaces. Hydrophilic surfaces allowed for the interchange of adsorbed albumin by extra-cellular matrix proteins [149,150] and greater osteoblast cell adherence. [151] Direct interactions involving the integrin receptors with the surface also transmit signals to control spreading and motility, modulating the formation of surface-mediated focal adhesion (FA). [152] Osteoblasts use integrin receptors to bind specific proteins adsorbed on implant surfaces (including fibronectin and osteopontin). [35,36,47].

The process of cell adhesion is specific in two ways: first, adhesive proteins mediating attachment may be specific for bone or a particular implant surface; and second, the osteoblast display of integrins is differentiation and matrix protein-specific.¹ For example, cultured osteoblasts use different receptors for attachment to polystyrene, titanium, and cobalt chrome alloy substrates. [17] These cell adhesion studies have directed attention to three main issues: composition effects, topography effects, and fabrication effects. [1] Topography deals with the roughness of the surface that should be described using discrete parameters. [153] The types of contacts observed in culture (focal contacts, focal adhesions, extracellular matrix contacts, and hemidesmosomes) and the principles that govern cell behavior (categorized as contact guidance, rugophilia, the two-center effect, and heptotaxis) must be studied in the context of physically defined implant surface characteristics. [154] A confounding factor in the analyses of surfaces of differing composition but of similar roughness is the physicochemical attributes of each surface. [63] Lastly, findings from cell culture attachment assays are not generally congruent with observations from *in vivo* studies. Electropolished surfaces, to which cultured cells avidly bind, do not support abundant bone formation *in vivo*. [155,156] In culture, smooth surfaces have been shown to promote attachment and spreading. These incongruent outcomes of *in vivo* and *in vitro* studies indicate that cell adhesion phenomena are necessary but not sufficient for osseointegration.

5.2.3. Cell Proliferation, Differentiation and Protein Biosynthesis Associated with Osteogenesis

Osteoblastic cell proliferation and differentiation are temporally opposing processes; undifferentiated cells proliferate rapidly, while differentiated cells divide rarely if at all. Surface-to-cell signaling also alters pathways that control proliferation. One example is the cross talk between integrin-signaling and the predominant MAP kinase pathways affecting cell proliferation. [157] Immediate and short-term cell culture can measure proliferative capacity and biosynthetic ability. These parameters are affected by different implant surfaces (experimental variables include defined composition and topography parameters). Cell proliferation is also largely influenced by the implant surface and is cell type-dependent. A reduced proliferation of fibroblasts compared to endothelial cells was observed in different types of implant surfaces. [158].

The gene expression pattern indicative of osteoblast differentiation is modulated by the implant surface. While the mechanism is largely undefined, the phenomenon is repeatedly observed. There is growing evidence from cell culture studies that the key regulatory mechanisms affecting osteoinduction and bone formation can be affected by surface topography. The impact of nanoscale surface modification includes elevated Runx2 expression (the key transcription factor controlling osteoblast differentiation). This is associated with orchestrated elevations in osteoblast-related gene (BSP, OPN, OCN) expression and ALP activity. [7-9,42,159] As stated previously, the majority of studies induce differentiation by means of culture media supplements. However, cell culture has provided a growing set of evidence that surface topography and related cell adhesion phenomena alter the differentiation pattern of adherent osteoprogenitor cells. The mechanism(s) acting to promote differentiation in a surface-dependent manner remains only partially defined.

The elaboration of a mineralizing matrix preceding bone is part of the bone forming process and occurs at the interfacial region surrounding endosseous implants. All mineralizing models include: (a) initial cell adhesion, (b) a proliferative phase, (c) a process of osteoblast differentiation, followed by the initial deposition (d) just before the onset of mineralization, (e) early mineralization; and (f) late mineralization stage. Although the lack of a more complete cellular environment is a limiting factor for cell culture studies; these models function as initial means of producing significant implant surfaces/bone interfaces hypotheses to be tested in vivo. Using the MG63 osteosarcoma cell line, different authors have shown that surface roughness enhanced osteoblast matrix production and enhanced cytokine expression. [67,69,98] Cell culture studies using a number of different cell models permit evaluation of individual steps in the process of matrix formation and mineralization. The surface also plays an important role in modulating the genes that modify collagen, such as Biglycan. [160-162]

5.3. Cell Culture and the Molecular Assessment of Osseointegration

The sequential process of osteoblast differentiation that underlies osseointegration has largely been attributed to the significant role of the mesenchymal stem cells and osteoprogenitors that populate the implant surface and interfacial region shortly after implant placement. Differentiation from stem cells to a functional osteoblast involves a series of different stages. The sequential and stage specific expression of osteoblast marker genes characterizes osteogenesis. Cell culture provides a convenient environment to monitor the influence of surface parameters on this cellular differentiation and extracellular matrix elaboration process of bone formation.

One of the central advantages of cell culture is that isolated aspects of complex biologic processes can be investigated in detail. Changes in cellular phenotype are often directly reflected in the pattern of gene expression at the mRNA level. In the cell culture environment, it is possible to address individual cellular responses to individual factors such as surface topography by evaluation of changes in the phenotype at the level of gene expression. The phenotype can be assessed globally by use of gene array technologies, can be directed at

specific physiologic pathways using smaller PCR arrays or can directly interrogate changes in single genes. All of these approaches have been applied to the study of osseointegration.

The process of osteoinduction and osteoblastic differentiation has been described in detail at the molecular level. The central effectors of osteoinduction include RUNX-2 and Osterix (OSX). RUNX2 or OSX gene ablation in mice results in a complete absence of bone skeleton. [163,164] The expression of these key osteoinductive transcription factors has been analyzed as a function of surface topography. RUNX2 and OSX expression are positively influenced by increased cpTi surface topography at the micron as well as nanoscale levels. [9,42,165-169] The gene expression of MSX2; a homeobox-containing transcription factor associated with proliferative osteoprogenitors has been studied on titanium substrates. [167] MSX2 is mostly expressed in proliferating cells with a gradual decrease during osteoblastic differentiation. [170] Its expression profile implicates that the proliferation phase is mainly associated with the first days of culture. Other significant transcriptional regulators include β -Catenin, and the homeobox-containing transcription factors MSX-1, MSX-2, DLX-3 and DLX-5. The addition of osteogenic supplements induces the differentiation of osteoprogenitors and mesenchymal stem cells with temporal expression of these various regulators. The impact of implant surface chemistry and topography on the expression (and activity) of these different central determinants of osteogenesis is the focus of several ongoing investigations.

Recent interest has expanded to include dental implant effects on osteogenic signaling upon the WNT pathway. Differential upregulation of several genes associated with Wnt signaling (CTNNA1, FBX4, FZD6) was identified by whole genome mRNA expression profiling of implant surface adherent primary human osteoblasts. [171] The potential role of the Wnt signaling pathway was in agreement with previous reports demonstrating elevated levels Dickkopf-1 (Dkk1) and Dkk2; factors that modulate the Wnt signaling pathway. [172-174] Silencing of DKK1 in osteoblast-like cells (MG63) abolished osteoblast maturation on microrough titanium surfaces. [172] Activation of WNT is a critical step in the differentiation and maturation of osteoblasts. [175].

Extracellular matrix (ECM) production represented by collagen type I, fibronectin (FN), vitronectin (VN) and tenascin have all been studied in the context of dental implant surfaces. Collagen type I represents the major protein component of the ECM, [176-178] and it is secreted early in the differentiation of stromal cells into osteoblasts forming the scaffold of the bone matrix. [179] The expression of mRNAs encoding these genes is influenced by the type of surface with higher expression levels on rough surfaces. [180,181] Furthermore, the expression of ALP, RGD-containing glycoprotein's such as BSP and OPN as well as OCN; a terminal osteoblast differentiation marker is enhanced with increasing surface topography. [42,92,100,165,167,168,178,181-184] Importantly, the orchestrated expression of these genes is necessary for the development of a mineralized phenotype. [185] Nanosurfaces were reported to induce earlier and elevated levels of expression of osteoblast related genes both in vitro and in vivo. [9,42] These are correlated with increased bone-implant contact and removal torque in animal models. [9,156] It appears that the modeled process of matrix protein expression and aspects of assembly and mineralization are well represented by cell culture performed on dental implant surfaces and that some of the observations parallel information obtained from in vivo studies.

The molecular mechanism by which implant surfaces alter the differentiation of cells in the osteogenic pathway is linked to the mechanisms of cellular adhesion (Figure 5–3 and 5–

4). Cells interact with their substrates via integrins. These adhesion receptors recognize binding domains within the proteins of the extracellular matrix. [186] $\alpha 2\beta 1$, $\alpha 5\beta 1$ and $\alpha V\beta 3$ represent the main RGD dependent receptors for COL1, FN and VN respectively. These integrins were also found to be prominently expressed by cells adherent to rough surfaces. [174,176,180,187] In cell culture involving dental implants, the implant surface is coated with plasma-derived ECM proteins such as Fibronectin. [35] Surface chemistry, surface energy and topography may directly affect the nature of the adhered ECM protein or, alternatively, directly influence signaling through various transmembrane receptors.

Integrin-mediated-adhesion to ECM proteins activates intracellular signaling proteins such as focal adhesion kinase (FAK) and SHC resulting in the formation of SHC-GRB2-complex. In turn, this activates Ras leading to stimulation of a cascade of signaling events involving the MAPK (mitogen-activated protein kinase). [188] The ultimate nuclear targets are members of the activated protein-1 (AP-1); c-fos and c-jun.

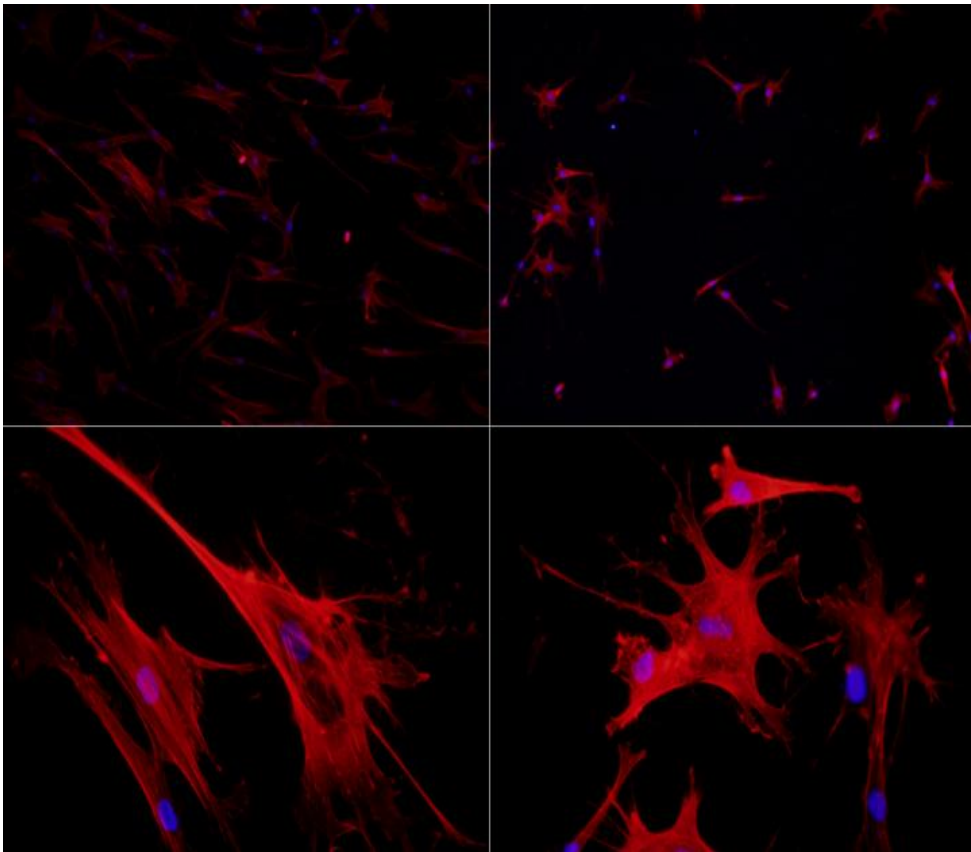


Figure 5.3. Cell adhesion is surface topography dependent. These immunofluorescent images reveal the effect of machined (isotropic) and grit blasted (anisotropic) surfaces on cell orientation and spreading. Left (machined, low and high magnification) reveals orientation of cell and stress fibers with the machining grooves. Right (grit blasted, low and high magnification) reveals no orientation and less extensive spreading of cells. Further investigation of the attachment-related signaling that affects osteogenesis is needed. After 24h cells were stained with Texas Red-conjugated Phalloidin (Invitrogen), which labels actin cytoskeleton (Red). The disks were mounted facing up on glass slides, and a glass coverslip was mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) (Blue).

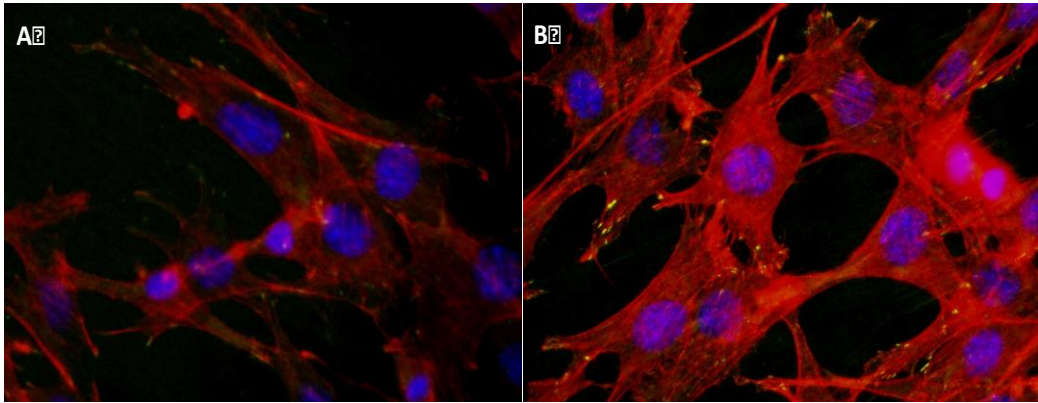


Figure 5-4. Focal Adhesions (FA) formation as cells differentiate into osteoblasts on cpTi surfaces. Cells were plated on disks and treated with (A) Regular or (B) Osteogenic media. The addition of osteogenic supplements to the media induced the formation of Focal Adhesions (FA). After 24h cells were stained with anti-paxillin (Invitrogen, Carlsbad, CA) (Green fluorescence). Followed by incubation with Texas Red-conjugated Phalloidin (Invitrogen), which labels actin cytoskeleton (Red). The disks were mounted facing up on glass slides, and a glass coverslip was mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) (Blue).

The promoters of several osteoblast related genes such as OCN and COL1 have AP-1 sites. [189] MAPK has been reported to regulate osteoblast differentiation. The extracellular-signal-regulated kinases (ERK) are a subfamily of the MAP kinases. ERK2 expression was increased more on the rough compared to smooth implant surfaces. [190,191] On the other hand, surface chemistry has been shown to alter phosphorylated ERK vs. total ERK demonstrating a possible mechanism of the surface altering osteoblast cell response through the ERK/MAPK pathway. [16] Using chemical inhibitors to block MAPK pathway signaling, the expression of Runx2-dependent osteocalcin expression was shown to be partially influenced by the addition of large titanium particles to the cell culture. Blocking the MAPK pathway using a MAPK inhibitor decreased osteoblast mineralization.

The process of osteogenesis is also associated with elevated expression of select growth and differentiation factors. Prominent among these are the Bone Morphogenetic Proteins. Elevated BMP2 expression is indicative of enhanced osteogenesis. Fluoride ion treatment of TiO₂ grit-blasted titanium substrates enhances the levels of BMP2 expression of human mesenchymal stem cells. [7] Most recently, the comparison of BMP expression in cells adherent to SLA and hydrophilic SLA (Mod SLA) titanium surface showed BMP2 expression, and to a lesser degree, BMP6 expression was greater on Mod SLA surfaces. Both surface and imposed hydrophilic modifications modify cellular responses that can be studied in culture. [171] The levels of transcriptional regulators of BMP expression (SMADs) are also modulated by surface-specific. [7,192].

Other factors that may play a role in osseointegration is VEGF. [193] VEGF is a molecule known to stimulate angiogenesis essential in wound healing. [194,195] VEGF also influences bone formation. VEGF levels were found to be elevated by titanium surface characteristics. [173,196] On the other hand, prostaglandin E₂ (PGE₂) and transforming growth factor- β 1 expressions have been analyzed. [69] Both were reported to be elevated on rough Ti-surfaces [167,176,197,198] and on hydrophilic surfaces. [140] TGF- β 1 plays an important role in wound healing and regulation of bone formation and bone resorption. TGF-

$\beta 1$ acts as autocrine regulator on the osteoblastic cells stimulating the replication of precursor cells of the osteoblastic lineage, in addition to their stimulatory effect on bone collagen synthesis. [199,200] On the other hand, TGF- $\beta 1$ acts via paracrine mechanisms to modulate the activity of osteoclasts. [201].

Recruitment of inflammatory cells, osteogenic cells and their progenitors are affected by differential expression of chemokines and/or their receptors. [202] In an effort to understand the factors involved in the recruitment of mesenchymal-like cells at the implant site, gene expression of several chemokines was studied in a rat model with different surface properties. [187] Among the genes that were modulated by titanium implants was CXCR4. CXCR4 is a chemokine receptor that plays an important role in homing and mobilization of different stem cell lineages including MSCs. [203-206] High binding of CXCR4 to its unique ligand stromal derived factor-1 (SDF-1) at the injury site ensure retention of the mobilized CXCR4-positive cells in the repair process. [207] Oxidized implant surfaces were associated with elevated levels compared to machined surfaces. [166,187] These studies reinforce the concept that multiple cell types populate the implant/bone interface and the investigation of these interfacial relationships is essential to a complete understanding of the osseointegration process. Many of these relationships can be studied in cell culture.

Bone accrual at the implant surface also involves resorption and remodeling. This process involves the balanced relationship of osteoblastic and osteoclastic cell types. Quantification of osteoclast-related genes may therefore represent a valuable tool to further understand tissue-implant interaction. Osteoprotegerin (OPG) plays an essential role in the control of bone resorption by acting as a decoy receptor of nuclear factor κB (RANKL); a molecule required for osteoclast differentiation. OPG functions to decrease osteoclast formation and activity. [208] Surface roughness and energy enhanced OPG expression levels in implant surface adherent osteoblast-like cells. [173,196,208,209] The understanding of osteoclastogenesis at endosseous implant surfaces requires further elucidation and the development of models that completely reflect the multiple cell types involved in this process.

Conclusion

The use of cell culture to investigate the process of osseointegration has expanded over the past 10 years to include models of osteogenesis as well as early steps of wound healing (platelet degranulation and inflammatory cell interactions). Although the modeled process of osteogenesis is largely dependent upon the use of osteogenic supplemented culture media, a large body of data demonstrates that surface characteristics (chemistry, topography) further influence the program of differentiation. Descriptive investigations of gene regulation provide evidence that substrate adhesion influences both short term and long term phenotypes of the culture system. Increasing substrate roughness increases osteoblast-specific gene expression. Only more recent studies have begun to probe the process of osseointegration at a mechanistic level. Examples include the use of gene knock-down and detailed investigation of transcriptional regulation. The linking of cell culture knowledge to in vivo observations at the molecular level offers further opportunity to improve the understanding of osseointegration and to direct change through targeted mechanisms.

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Chapter VI

Implant Prosthodontics: *In Vitro* Testing Methods

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ABSTRACT

While randomized controlled clinical studies are strongly suggested as the optimal approach to evaluate the performance of biomaterials and design aspects of dental implants and prosthetic components, these studies are at times not economically viable. Thus, well designed *in vitro* studies associating virtual models via finite element analysis (FEA) and appropriate *in vitro* laboratory mechanical testing should be utilized as preliminary investigative tools to reduce the number of clinical studies that are needed to fully characterize the performance of a given implant-restorative system.

The present chapter describes the computer simulation using FEA and *in vitro* tests as single-load-to-failure, staircase and step-stress accelerated life-testing utilized to evaluate implant-restoration systems with emphasis to the advantages and potential limitations of each methodology and how the acquired outcomes can be related to the clinical scenario. Major issues and approaches related to the FEA, such as model creation, finite element software, material properties, bone-implant interface, mesh and

convergence of the analyses, boundary and loading condition, validation and interpretation of FE modeling and criteria to interpret the results in FEA studies are listed and described.

6.1. Introduction

Several reports have emphasized the integrity of implant-prosthetic components and endorsed the long-term effectiveness of osseointegration. [1-4] However, despite the high success rates reported, [5-9] failures of implant-supported rehabilitations are still unavoidable due to systemic (i.e. diabetes mellitus), biological and/or mechanical complications.

From a mechanical stand point, forces occurring either from functional or parafunctional occlusal contact may result in a physiologic adaptation of the supporting tissues since implants are rigidly anchored to the bone. However, if the stress generated is beyond the adaptive capacity of the host, the response of the supporting tissues and prosthetic components may result in failures. Therefore, the load magnitude and duration employed to implant-restoration systems play a significant role in the stress transferring to the implant-prosthesis and living tissues. [10].

While prosthetic connection failures have been reported in all regions of the mouth, its higher incidence mainly concerns the posterior region. [11,12] The most commonly observed mechanical problem has been associated with screw joint instability, including screw loosening as well as abutment and /or screw fractures. [13-16] For instance, an incidence of 7.3% of screw loosening or fracture has been reported after 5 years of service. [17] Although implant fracture has been infrequently reported, [18] the literature has underestimated its prevalence since follow-ups of most of the studies are generally limited to 5-7 years. [19].

While randomized controlled clinical studies are strongly suggested [20-22] as the optimal approach to evaluate the performance of biomaterials and design aspects of dental implants and prosthetic components, these studies are at times not economically viable. Thus, well designed *in vitro* studies associating virtual models via finite element analysis (FEA) and appropriate *in vitro* laboratory mechanical testing should be utilized as preliminary investigative tools to reduce the number of clinical studies that are needed to fully characterize the performance of a given implant-restorative system. [19].

The present chapter describes the computer simulation using FEA and *in vitro* tests utilized to evaluate implant-restoration systems with emphasis to the advantages and potential limitations of each methodology and how the acquired outcomes can be related to the clinical scenario.

6.2. Finite Element Analysis (FEA)

This section describes some of the major issues and approaches involved in the steps to design a FEA. FEA is a numerical tool for obtaining a solution to a given mechanical problem by dividing the problem domain into a collection of much smaller and simpler domains in which the field variables can be interpolated with the use of shape functions. [23-25].

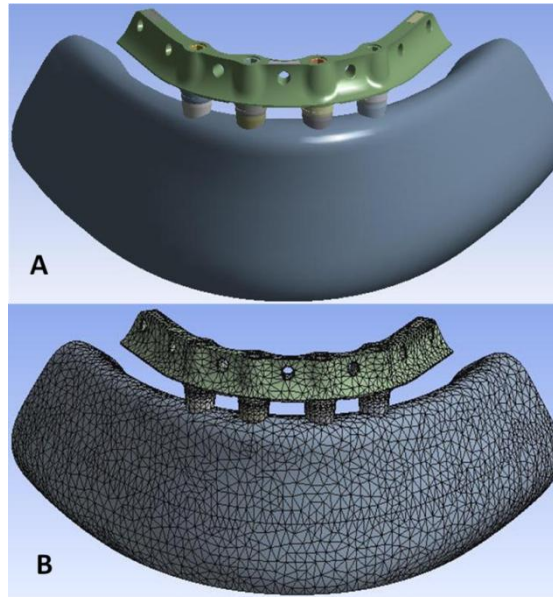


Figure 6.1. A - Model of completely edentulous mandibular arch with multiple implant-superstructures; B - Finite element mesh of the model.

This method allows the researcher to address a range of questions that are otherwise intractable or very difficult to solve analytically [25] and is potentially one of the most powerful tools in the methodological arsenal for implant-supported prosthesis therapy.

In mechanical terms, the application of a load results in stresses and strains in the structure. [26] Stress (σ) is defined as force per unit area (F/A) and describes the internal forces in an object. [27].

Strain (ϵ) describes the deformation that results from an imposed load and is defined as the change in length divided by original length. By customary mechanics of materials convention, stretching in tension is a positive strain and compression is a negative strain. [26].

Stress and strain may be solved exactly by analytical means for simple geometric shapes with homogeneous material properties. However, complex problems are computationally impractical or intractable. [28] Even a simple geometry cannot be solved analytically if the material properties or loading conditions are graded or complex as commonly observed in biological structures. The finite element method provides an approximate solution to such problems by subdividing the complex geometry into a finite (but typically high) number of elements of simple geometry (Figure 6-1A and B). [26].

In numerical analysis, the idealization of the physical problem to a mathematical model requires assumptions that lead to differential equations that rule the model and, since the process is numeric, it is essential to verify its predictive accuracy. [29].

Based on its versatility, FEA has been used to investigate different therapeutic strategies in the treatment of the complete and partially edentulous scenarios with implants and their importance for the treatment outcome comparing stress distribution on single implant-supported restorations, [30-34] implant-retained overdentures [35,36] and multiple implant-supported superstructures. [37-43].

Major issues and approaches, such as model creation, finite element software, material properties, bone-implant interface, mesh and convergence of the analyses, boundary and

loading condition, validation and interpretation of FE modeling and criteria to interpret the results in FEA studies are listed and described as follows.

6.2.1. Model Creation

The first step in creating a finite element (FE) model is to decide the dimension (1-, 2-, or 3D) of the problem. Greater dimensions, while potentially more realistic, are proportionally more difficult to model, solve, and subsequently examine. [26] For selected situations, the 2D analyses are often adequate for the questions at hand. [36, 44-46] The choice between 2D and 3D FEA to investigate the biomechanics of complex structures depends on many factors, including the complexity of the geometry, the type of analyses required and expectations in terms of accuracy, and the general applicability of findings. [47].

The 2D FEA has been extensively used in different areas of dental research. [45,46,48] However, it is assumed that 2D FEA has limitations, which may compromise the accuracy and reliability in determining the mechanical behavior of different designs of dental restorations. [49,50] In contrast, although 3D FEA may offer acceptable reliability and can fully capture the geometry of complex structures, the complexity of 3D FE models often makes it difficult to achieve the same mesh refinement and hence numerical accuracy as in 2D models. The advantages of employing 3D FEA must be weighed against the difficulties in modeling and preparing such models for mesh generation analyses. [47].

Accurate 3D reconstruction is essential to study the interaction between morphology and mechanical behavior as well as to help in the design of specific prosthetic components and implants. [51] A variety of techniques are available, including manual and automated techniques. The choice for building a model with manually or automatically technique depends on the purpose of the study and the structure of interest.

The manual technique is based on the actual dimension of the structures obtained from the literature for building the model in a CAD software as AutoCAD (Autodesk Inc, San Rafael, CA, USA), SolidWorks (SolidWorks Corp., Concord, MA, USA) or Pro/Engineer (Wildfire, PTC, Needham, MA, USA) among others (Figure 6-2).

The automated approach involves transforming laser scans of bones or other structures into wireframe models that are then converted into FE models. [47] Laser scans can offer a high-resolution representation of the outer surface but lack information about internal geometry. [52] Micro-tomography dataset can also be directly transformed in a high-resolution FE model by using specific software such as Mimics (Materialise, Leuven, Belgium) (Figure 6-3). [53] The automation and high resolution make this method attractive, but it also involves complex issues in finding appropriate thresholding algorithms to demarcate the bone-air boundary reliably throughout a structure with varying bone thickness and density. [54]

Independently of the software or technique to build a FE model, the researcher should apply the St. Venant's principle to define the size of the model. The principle establishes that the strains produced in a body are of negligible magnitude at distances from the main point of interest. Based on this principle, for instance, does not make sense to use an entire mandible or maxilla to study the bone behavior close the first treads of an implant. Small models should be mathematically more appropriate for that. In addition, small models will improve the analysis by using a predictable refined mesh to achieve the purposes accordingly.

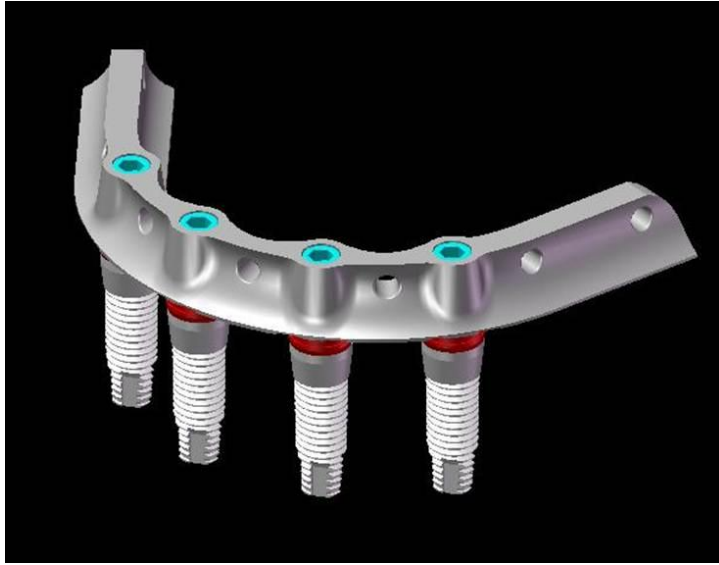


Figure 6.2. Prefabricated mandible bar-prosthesis protocol building in the SolidWorks software (SolidWorks Corp.).

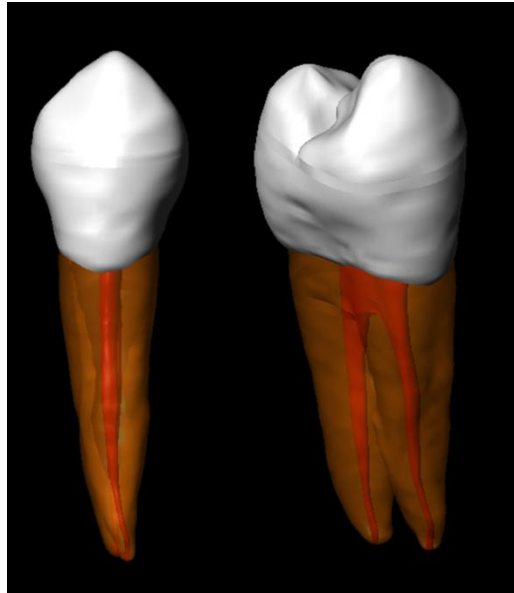


Figure 6.3. Lower first pre-molar built using Mimics Software (Materialise) from the Micro-tomography (μ CT) slices (CT40, Scanco Medical).

6.2.2. Finite Element Software

The most common software reported in the literature for prosthetic/implant FEA are ANSYS (Swanson Analysis Systems, Houston, Pa), [37,38,45,55-60] ABAQUS (Pawtucket, RI, USA), [61-64] COSMOS (Structural Research and Analysis Corp., Los Angeles, CA, USA) [65,66] and MSC PATRAN (Santa Ana, CA). [67].

When the model is finalized in the CAD software, *igs.* files are transferred to one of the finite element programs described above containing the material properties, boundary and loading conditions and the characteristic of the contact interface between all the materials. The software utilizes all these information to understand the material behavior when it is submitted to deformation under determined stress fields.

6.2.3. Material Properties

Material properties such as heat conductivity, linear and nonlinear elastic properties, and temperature-dependent elastic properties can be modeled for FEA. [26] In dentistry, the majority of the FE articles in implant prosthodontics consider the material properties as isotropic, homogeneous and linearly elastic to simplify the mathematical calculations. [68,69] An isotropic material indicates that the mechanical response is similar regardless the stress field direction, requiring Young's modulus (E) and Poisson's ratio (ν) values for the FE calculation. [26].

The elastic, or Young's modulus (E), is defined as stress/strain (σ/ϵ) measured in simple extension or compression. It is a measure of material deformation under a given axial load; in other words, a numerical description of its stiffness. Poisson's ratio (ν) is the lateral strain divided by axial strain, thus representing how much the sides of a material contracts as it is tensed (or, conversely, how the material will expand as it is compressed) to maintain its volume. [26].

However, as bone is one of the structures to be simulated in FEA, it should be considered as an anisotropic structure since its mechanical properties depend on various factors such as porosity, level of mineralization, density, organization of the collagen fibers, speed of deformation and direction of the load. [38,70,71].

The literature has considered cortical bone with a higher (E) in the axial direction compared to radial (perpendicular to the surface) or tangential (parallel to the surface) orientations. [72] Based on this configuration, the cortical bone is simulated as an anisotropic structure for the FEA and three elastic modulus (E), three Poisson's ratio (ν) and three shear modulus (G) are used. [73,74] These values are characterized by different stress responses under forces applied in varied direction. [75,76] However, the elastic modulus (E) in most cortical bone approximates to orthotropic, which is a type of anisotropy in which the internal structure of the material creates unique elastic properties along each of the three orthogonal axes of the material. In this case, three elastic (E) and shear modulus (G) and six Poisson's ratios (ν) are used. [26] Orthotropy is not in itself a problem for the FE method. However, the cross-sectional shape of the mandible does not easily lend itself to the use of orthotropic material properties, for which the symmetry axes would presumably change from point to point, following the irregular elliptical shape of the mandibular cross section. [76] In addition, the bone quality measures the structural efficiency of the extracellular matrix or architecture of the trabecular bone. Thus, scenarios of poor bone quality presents a critical limitation for the assessment of bone integrity when compared to the bone with complex structure. [71] For instance, clinical studies have revealed that dental implants placed in regions of the jawbone with lower density have a higher chance to fail than implants placed at regions with higher bone density. [77].

Table 6-1. Mechanical properties of the most used structures in implant prosthodontics research through finite element analysis

Material	Young's Modulus (GPa)	Poisson's Ratio	References
Bone type I	9,5	0,3	[80]
Bone type II	5,5	0,3	[80]
Bone type III	1,6	0,3	[80]
Bone type IV	0,69	0,3	[80]
Cortical bone	13,70	0,3	[60]
Titanium	110	0,35	[68]

There is no consensus between the authors about the elastic model (E) of trabecular bone. The values range from 0.3 to 9.5 GPa. [32,39,78,79] However, Tada and coworkers [80] identified the type of modulus (E) according to the type of bone - bone type I (9,5GPa), bone type II (5,5GPa), bone type III (1,6GPa) and bone type IV (0,69GPa) - and these values have been used by other researchers depending on the region where the model was generated (Table 6-1). [38].

Considering the fact that the most common bone quality types in mandible are type I or II in the anterior bone and type III in the posterior region, [81] the elastic modulus (E) that would best represent the mandible would be 9.5 GPa or 5.5 GPa for the anterior region and 1.6 GPa for the posterior region. For the maxillary bone, the most prevalent types of bone are type II or III in the anterior region and type IV in the posterior region. Therefore, the Young's modulus that would best represent these sites would be 5.5 GPa or 1.6 GPa for the anterior region and 0.69GPa for the posterior region, respectively. For cortical bone, studies typically use the elastic modulus (E) of 13.7 GPa and the Poisson ratio (ν) similar to 0.3 for the trabecular and cortical bone (Table 6-1). [32,68].

6.2.4. Bone-Implant Interface

In implant therapy, the fact that the biomechanical reaction of the bone differs for each patient implies that it is a challenge to model the percentage of osseointegration accurately. A fixed bond between the jawbone and implant along the entire interface has been commonly assumed. [10,82,83] This non-linear condition considers that the structures are 100% integrated, which indicates that the trabecular and cortical bone are perfectly bonded to the implant interface. [69,84] In addition, bone is commonly treated as a linearly elastic material based on its elastic properties in response to physiologically normal loads. [26] However, this assumption does not appear realistic for the clinical conditions where bone loss or remodeling could be presented. [85] Currently, many existing FEA packages offer a variety of frictional contact algorithms to simulate different bonding conditions at the bone-implant interface. [68,86,87] Mellal and coworkers [88] and Pessoa and coworkers [31] have previously considered 0.3 as a possible frictional contact. Huang and coworkers [68] considered three different frictional coefficients (0.3; 0,45 and 1) to simulate the contact between implant and bone. Nonetheless, the interfacial frictional characteristics have to be determined experimentally and then utilized as property input for FEA. [68,89].

6.2.5. Mesh and Convergence Analysis

Transforming the geometry of a physical structure into a FE model involves subdividing it into a finite, but usually large, number of geometrically simple domains called "finite elements" that are connected together at their vertices called "nodes". The resulting contiguous collection of simply shaped elements connected together by their nodes is called "mesh". A node is a coordinate location in space where degrees of freedom are defined and the displacements are determined in response to the loads. [26].

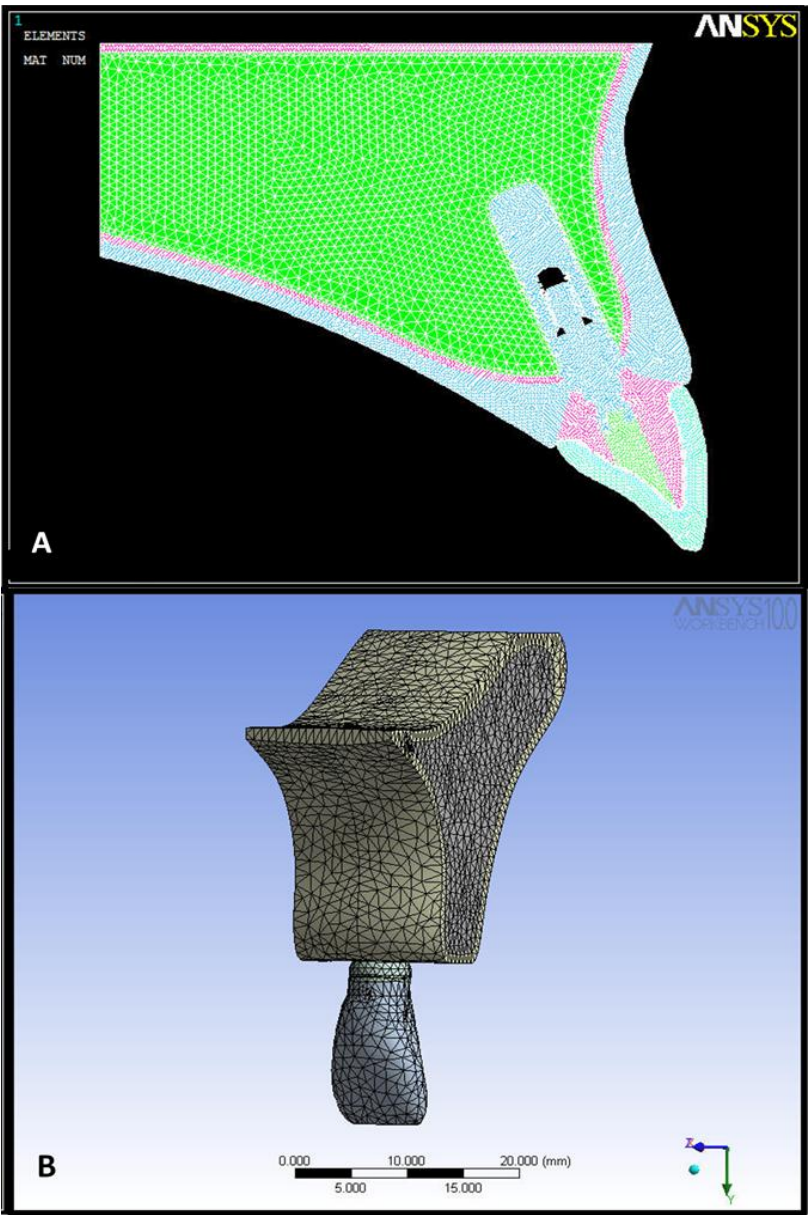


Figure 6.4. A - 2D Finite element model meshed with triangular elements; B - Finite element 3D model meshed with tetrahedral elements.

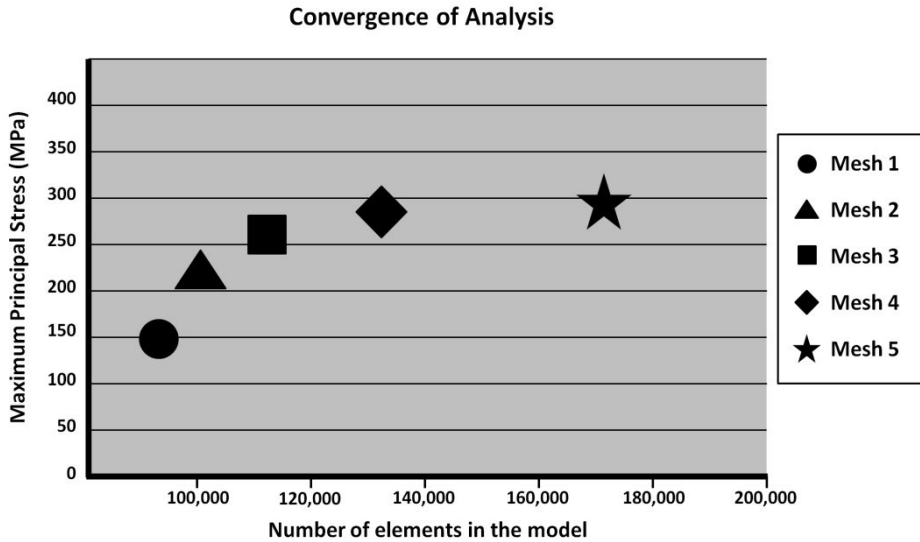


Figure 6.5. Maximum principal stress ($\sigma_{\max}$) in hypothetical convergence testing of mandible FE models meshed by 0.6 mm elements with no refinement (mesh 1) and with refinement levels 2, 3, 4 and 5 at the more posterior implant near the crestal region (meshes 2, 3, 4 and 5, respectively).

Since the components in a dental implant-bone system are extremely complex from a geometric standpoint, FEA has been viewed as the most suitable tool for analyzing them. A mesh is needed in FEA to divide the whole domain into elements. The process of creating the mesh, elements, their respective nodes, and defining boundary conditions is called “discretization” of the problem domain. [23].

The 2D structures are typically meshed with triangular or quadrilateral elements (Figure 6-4A). These elements may possess two or more nodes per side, with one node at each vertex. If nodes are only placed at the vertices, the element is called linear because a line function describes the element geometry and how the displacement field will vary along an element edge. [26].

Typical 3D elements include eight-node bricks, six-node wedges, five-node pyramids, and four-node tetrahedral (Figure 6-4B). With increasing numbers of elements and nodes, the model becomes more complex and computationally more difficult and lengthy. [90]

To address this problem, researchers often devise strategies to minimize computational expense, such as taking advantage of symmetry when possible by modeling only half the structure, using a generally coarse mesh with finer elements only near regions of geometric complexity or high stress and strain, and/or using a 2D model when it suffices. [26]

A convergence study of the model is always important to verify the mesh quality. A measurement of convergence is the degree of difference in the total strain energy between two successive mesh refinements. When the difference in energy is less than some tolerable limit specified by the user, the solution is considered converged (Figure 6-5). Convergence is a function of the type of element model employed, the applied loads, the equivalent loading transformation and the degree of subdivision. [91] Some authors considered that the convergence criterion between meshes refinement was a change of less than 6% in the maximum simulated maximum principal stress ($\sigma_{\max}$) of the bone-implant edge (Figure 6-5). [68]

6.2.6. Boundary Condition

Most FE studies only considered a small part of bone surrounding the implant. [80,92] It can be explained by the Saint Venant's principles, which the actual force system may be replaced by a statistically equivalent load system and the distribution of stress and strains is only affected near to the region of loading.

It has been common to apply fixed constraints to the lower region of the jawbone during the construction of the finite element model of jawbone to simulate the continuity of the mandibular ramus and to avoid inertial movement (Figure 6-6A and B). [83] These fixed support represent the constrained of x , y and z directions (displacement = 0).

Ishigaki and coworkers [93] determined the directions of displacement constrains, which were applied to the jawbone accordingly to the angles of the closing pathways of chopping type and grinding type chewing patters. The models were restrained at the base of the maxillary first molar to avoid sling of the entire model. Okumura and coworkers [94] simulated a posterior maxilla with an implant and determined that the models were constrained in all directions at the nodes on the mesial and distal bone surfaces, the top on the simulated sinus and upper half of the sinus walls. Shunmugasamy and coworkers [60] simulated different macro-geometries of unit implants and fixed the base of the outer sides of the cortical bone. Teixeira and coworkers [95] considered that as the strains dissipated sharply in a small volume around the implant, these remote boundary conditions did not have an impact on the analysis results.

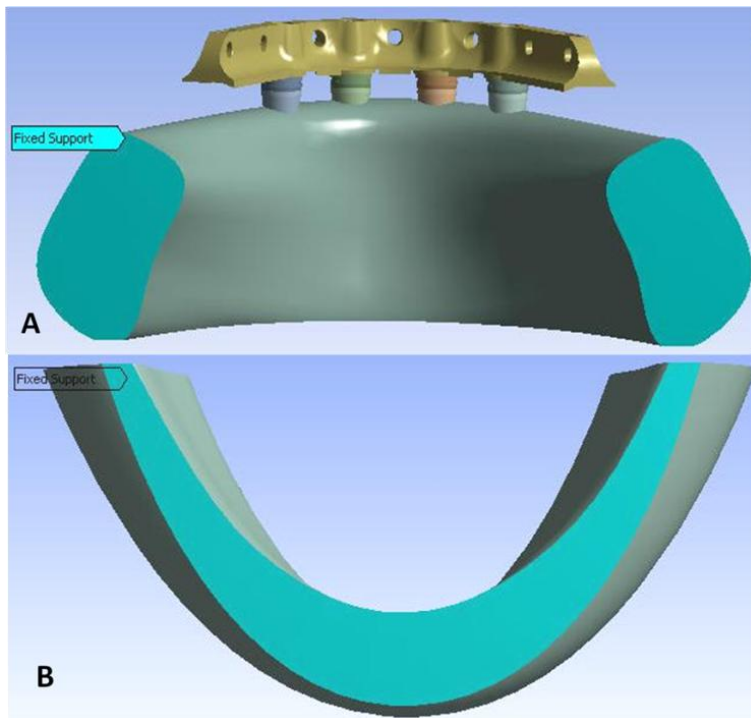


Figure 6.6. Boundary conditions of the complete edentulous mandibular arch at the posterior region of the jawbone to simulate the continuity of the mandibular ramus (A) and at the inferior region of the mandible to void inertial movement (B). The green region represent the fixed support described above.

6.2.7. Loading Conditions

In order to produce accurate predictions of the implant, it is essential to determine realistic loading magnitudes and directions. [83] Several studies have also suggested that loading an implant at low magnitude of force can promote early peri-implant osteogenesis. [86,96,97] One of the advantages of early loading is that the time required for healing can be shortened dramatically to benefit the process of bone remodeling.

The combination of axial and transverse loading, termed as mixed loading, simulates practical conditions where the actual applied force may be inclined with respect to the implant axis and components can be solved in the longitudinal and transverse directions (Figure 6-7). [60].

Several investigations have assumed the directions of the load applied to the implant to be in three directions (horizontal, vertical and oblique). Canay and coworkers [98] and Tada and coworkers [80] assumed a horizontal load of 50N and a vertical load of 100N with no oblique force whereas Meijer and coworkers [99] assumed a horizontal force of 10N, vertical force of 35N and an oblique force of 70N at an angle of 120° from the horizontal.

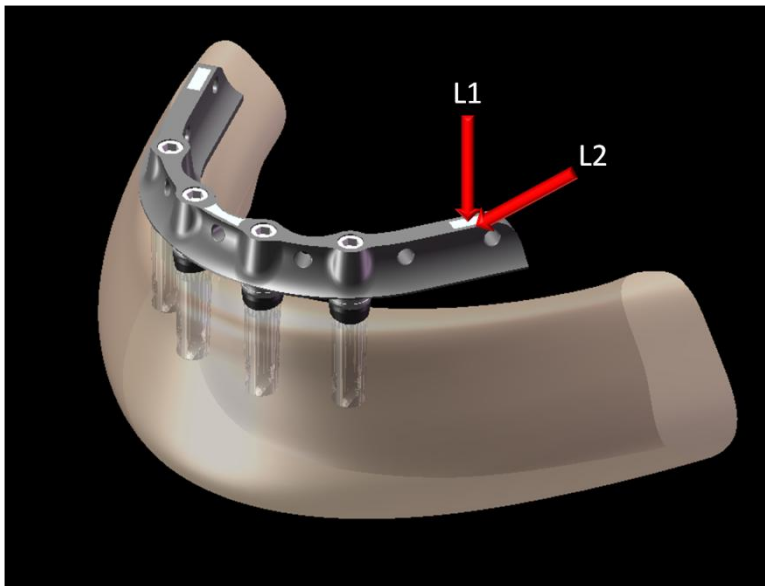


Figure 6.7. Axial (L1) and oblique (L2) loading being applied on the implant superstructure cantilever.

6.2.8. Validation and Interpretation of FE Modeling

The validation of the results is the final step in the FEA and is the most important phase, depending on the degree to which material reflects its biological reality. [26] For biological problems, researchers should be able to address both the precision and accuracy of the model. [100] Thus, precision is similar to its use in statistics, where it relates to the number of significant figures recorded for measurements. [101].

A model would be precise but inaccurate if the mesh is very dense but the loading and boundary conditions are unrealistic. Precision and accuracy require different kinds of

validation. In the past, assessing precision was an important step since limitations on computer power required researchers to determine an appropriate degree of mesh refinement that was reasonably precise but minimized computational demand. This has become less important since more powerful computers allow programs to automatically generate meshes that typically have ample density and increase mesh density in regions under high stress concentrations. However, the only way to assess discretization error is to conduct a convergence test in which specific analytic quantities at specific locations are compared between three or more meshes of different refinement. [26].

Validation of FEA entails comparing the behavior of the model with *in vivo* or *in vitro* data gathered from parts of the modeled structures. A combination of *in vitro* and *in vivo* experimentation potentially offers the best validation. *In vitro* validation allows one to carefully control the loads and boundary constraints in order to assess the validity of the model's geometry and elastic properties. [26] Studies of the consistency of numeric models and their agreement with biologic data are scarce [88,102] and the level of agreement and consistency between different engineering methods, especially regarding quantified stress/strain, remains a concern. [103,104].

6.2.9. Analysis Criteria

The analysis criteria widely used in implant prosthodontics are equivalent von Mises stress (σ_{VM}), [30,31,34-37,40-43,68,69] maximum and minimum principal stress, (σ_{max} and σ_{min}) [37,38,105] and maximum and minimum principal elastic strain (ϵ_{max} and ϵ_{min}). [31,33,34,37,69].

Although the majority of the studies have used the von Mises principal stress (σ_{VM}) for evaluation of implant prosthodontics stress, several researchers suggest that the magnitudes of the concentrations should be presented in the maximum and minimum principal stresses (σ_{max}) for evaluation of stress distribution in a brittle structure as bone. [38] This analysis criterion offers the possibility of making a distinction between tensile stress and compressive stress. In addition, displacement components of specific points provide information about the deformation of the model and facilitate interpretation of the results. Principal stress values of fragile compact bone can be compared with its ultimate compressive strength and ultimate tensile strength values. [106,107].

In fact, interfacial failure and bone resorption under different stress types are attributed to different mechanisms. Accordingly, it may be erroneous to emphasize the peak compressive or von Mises equivalent stresses without considering the risks of the tensile and the shear stresses at the interface. [105] However, when the titanium component is available as abutment, screw and implant, which are ductile structures; the von Mises Principal Stress, that is an stress mean, is a recommended analysis criterion. [65].

Considering the FEA characteristics described above, the main advantage is the virtual simulation of real structures that are difficult to be clinically evaluated, i.e. stress and strain distribution on periimplant bone. Moreover, it is a low cost alternative in comparison to other *in vitro* methods since only a virtual model is used. However, some clinical parameters cannot be reproduced in FEA, such as wet environment and damage accumulation under repetitive loading. Thus, *in vitro* tests are recommended to complement the FE results and described below as another way of simulating oral environment conditions.

6.3. Mechanical Testing

The treatment success of implant-supported prosthesis is dependent on stress transferring through prosthetic components, implant and supporting tissues. Although several studies utilize single-load-to-failure to obtain strength data, [108] fatigue-testing methods have demonstrated to be the most adequate and clinically relevant modalities to evaluate mechanical response of implant-prosthesis systems. [109-111] Fatigue is partially or totally related to failure, and the incidence of these failures usually increase with the longer the implant and prosthetic components loaded, demonstrating that fatigue and subsequent failures is a time-dependent phenomenon. [109]. It is important to highlight that several devices are available for repeatedly placing a specimen under stress condition during a fatigue testing. However, considering that the test accuracy depends on simulation of real clinical conditions, some parameters should be controlled; such as cycling frequency, stress amplitude, dry/wet ambient, temperature, and reproduction of multidirectional functional loading. In addition, the application of engineering concepts is crucial to understand the stress concentration and distribution of implant-restoration materials.

6.3.1. Single-load-to-failure

The single-load-to-failure (SLF) or ultimate fracture strength (UFS) test is characterized by loading of the specimen through compression until failure at a constant strain rate. During the test, a force/displacement curve is acquired for each specimen and the maximum load to failure is recorded (Figures 6-8 and 6-9). [108].



Figure 6.8. An off-axis compressive load applied on the occlusal surface of a molar crown during single-load-to-failure test.

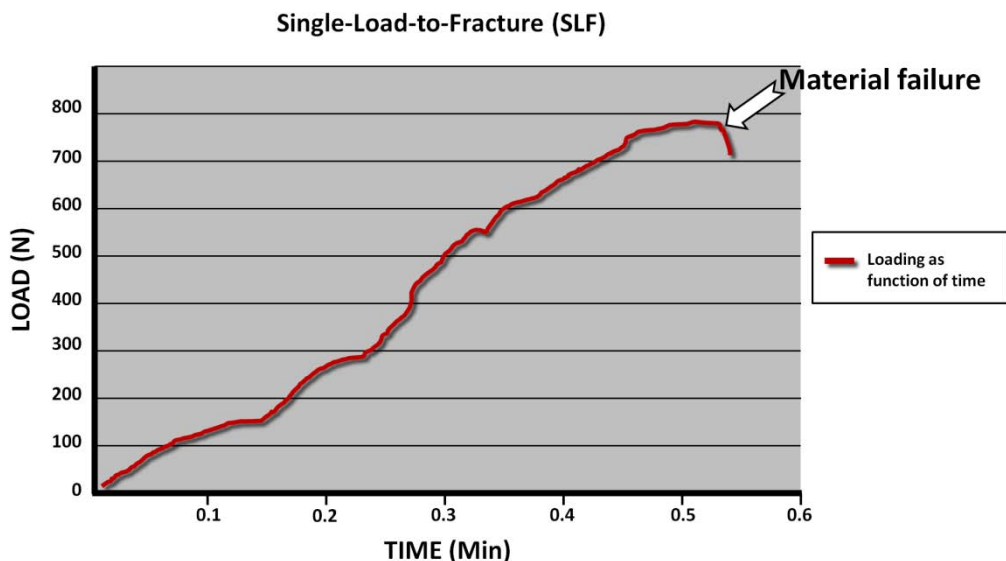


Figure 6.9. Chart depicting *Load vs. Time* curve generated by Compression Method test until failure of the specimen as illustrated in Figure 6.8.

Considering that this test approach consists of loading the samples at stress levels higher than use stress in order to facilitate failures in a timely manner, [109] *in vitro* studies have applied such technique to evaluate the strength of prosthetic components of implant-supported prostheses. Andriani and coworkers [108] used this mechanical testing to evaluate the strength to failure and fracture mode of three indirect composite materials directly applied onto titanium implant abutments and cemented porcelain-fused-to-metal crowns. In this study, the specimens were loaded to failure in compression by applying a ramping force in the tip of one of the four cusps by means of an indenter (10 mm x 2 mm rectangular shape) at a rate of 1 mm/min in a universal testing machine (Instron 5666 machine, Canton, MA, USA). After failure, the fracture mode of the specimens was classified according to the crack propagation direction. Although the single-load-to-failure test is an easy technique to measure the maximum fracture strength of a component/material under compression, it has a limitation in simulating the cyclic intraoral function. Moreover, in an oral environment the implants and restorations are not frequently submitted to increasing forces until failure, but to a cumulative damage with high and low forces generated repeatedly during mastication. The significance of this statement was demonstrated in the study of Coelho and coworkers [112] when anatomically correct veneered zirconia crowns exhibited a single-cycle load to failure of $1227 \pm 221\text{N}$ but only a 48% (23-68% at 90% confidence) probability of surviving when a 200N load was applied for 50,000 cycles. Thus, several studies [109-114] have applied the single-load test method only to obtain the mean load of fracture, so that the failure behavior of given specimens can be determined for subsequent fatigue tests.

6.3.2. Fatigue

The term “fatigue” was first proposed by Panalet in 1839, a time when the industrial revolution had started and rapidly moving parts became increasingly common. The main line

of thought explained fatigue fractures by "crystallization" of the material which became brittle after continued use and thus more prone to fracture. [115] Fatigue is a mode of failure whereby cracks are induced by subjecting a material or structure to repeated sub-critical loads, which eventually leads to failure. [115].

The fatigue resistance of a component is usually assessed by an S-N diagram in which "S" is the stress and "N" is the number of cycles endured before failure. [116] Among the several techniques suggested for this analysis, most are based on quantal data, which means that the analysis is based on the number of "fail" or "survival" results at a given level of stress. [117].

In the dental field, fatigue failure results from the development of microscopic cracks in areas of stress concentration that, under continued loading, fuse to an ever growing fissure that weakens the restoration. [115] This initial step is termed nucleation and represents a mandatory stage of fatigue failure. When a fissure reaches its critical size, it will definitely progress at each loading cycle. This process is referred to as propagation and amounts to about 90% of fatigue life. At the end, catastrophic failure occurs as a result of final loading cycle exceeding the mechanical capacity of the remaining sound portion of the material [115].

Considering that the failure in implant-supported restorations is usually associated to damage accumulation generated by functional loading, the fatigue test methods are alternatives to assess the behavior of implant-prosthesis assembly. There are commonly three fatigue methods commonly used for dental research: constant stress, staircase, and step-stress. Regardless of the *in vitro* method used, there is the need for substantial preclinical evaluation of implant-supported prosthesis prior to its release to clinical trials. Thus, methods capable of predicting clinical performance over time are highly desirable. Advantages and disadvantages of each of the most commonly used three methods are described below.

6.3.3. Constant Stress Test

When a material is cyclically tested under a constant loading, a constant stress test is characterized. In order to simulate human masticatory function, several *in vitro* studies [118-123] in implant prosthodontics run the specimens at constant stress (Figure 6-10).

Assunção and coworkers [118] subjected single implant-supported prostheses to mechanical cycling with constant loads of 130N attempting to simulate loading conditions in the anterior region of the mouth. Similarly, the dynamic fatigue properties of the dental implant-abutment interface were simulated by using constant stress by several other groups. [119-123].

According to the literature, [117,118,121] an individual presents three episodes of chewing per day during 15 min at a frequency of 60 cycles/min (1Hz), resulting in 2700 cycles/day or almost 10^6 cycles during 1 year. However, to date, the *in vitro* constant stress test protocols vary among studies, making it difficult to compare results since there is no consensus about the number of test cycles vary from 10^3 to 10^6 and up, [124] and the frequency (Hz or cycles per second) for load application. The last has also varied among previous studies (2 Hz, [118] 6 Hz, [121] 8Hz, [120] 10Hz, [122,125] 14 Hz, [123] and 15 Hz [119]).

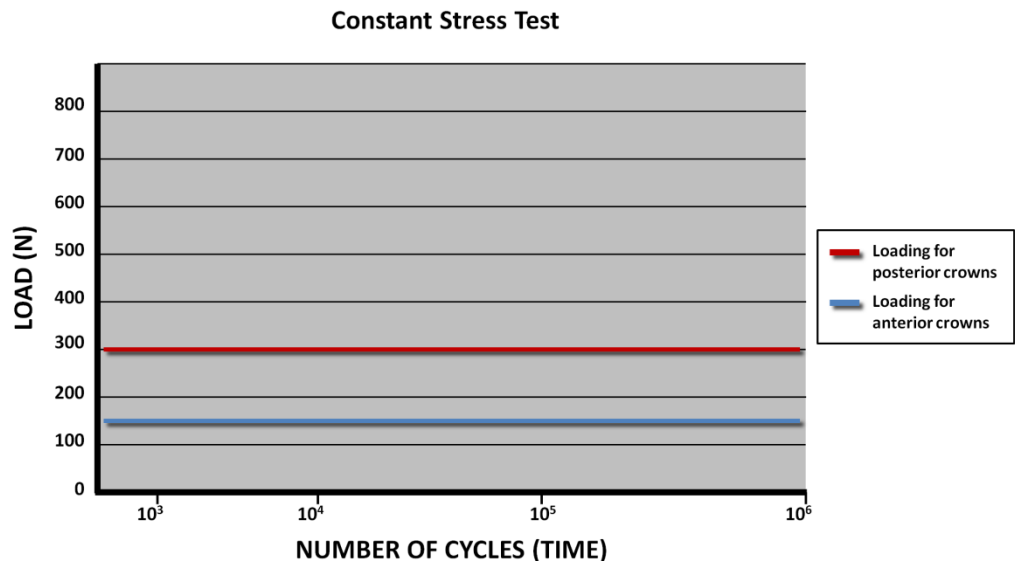


Figure 6-10. Constant magnitude of load applied to estimate life expectancy of posterior and anterior crowns usually employed in implant-prosthesis studies.

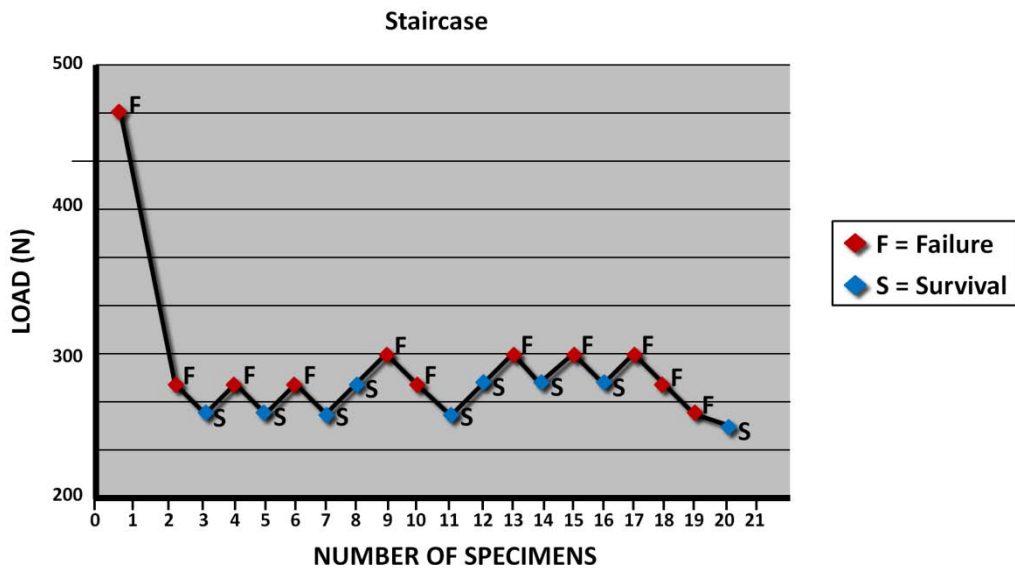


Figure 6-11. Chart representing a *Staircase Test* of 20 specimens subjected to constant fatigue until failure (F) or survival (S) after a predetermined number of cycles. Note that when a specimen fails, the next specimen is tested at a lower stress level. On the other hand, if the specimen does not fail, the following test runs at a higher stress level.

Thus, there is the need for further investigation concerning the parameters that should be employed in this method and its correlation with clinical failures. How this method is performed and potential limitation regarding the fact that the samples need to be further fractured after fatigue and what the implication of this issue would be.

6.3.4. Staircase Test

The staircase method, is a straightforward procedure in which a series of samples is tested consecutively to determine statistical properties of a fatigue limit. [117,126,127].

The test is conducted in such a way that each specimen is tested during a determined lifetime corresponding to the infinite life. Although a life range from 10^6 to 10^8 cycles has been suggested, [115,128] the number of cycles for which the component's fatigue strength will be determined can be guided by previous experience and based on the expected number of cycles the component is likely to be subjected during its intended life.

In the staircase method, if the specimen fails prior to infinite life, the next specimen will be tested at a lower stress level. If the specimen does not fail within this life of interest, the subsequent sample will be tested at a higher stress level. Therefore, each test is dependent on the previous specimen result and it will be conducted in sequence with the stress level being increased or decreased by selected stress increments. It is recommended to run the test with at least 15 to 20 specimens (Figure 6-11). [126,128].

Considering that the test will only begin when the first turnaround occurs, extra samples can be necessary to adequately set the initial stress amplitude for the procedure, [126,128] which represents a cost increase for the experiment. In this case, the SLF test can be previously applied as a pilot study to determine the entry stress level and avoid fabrication of additional specimens. At the end of the staircase testing sequence, data reduction techniques must be applied to determine the statistical distribution of the staircase test results, such as the Dixon and Mood, and Zhang and Kecioglu methods. [126].

The Dixon and Mood method is based on the maximum likelihood estimation (MLE) and provides calculation of mean and standard deviation of a fatigue limit when it follows a normal distribution. This approach considers either only the failures or only the survivals to determine the statistical properties and is dependent on the least frequent event. The method was proposed at a time when computing power was a scarce resource and simple approximate methods were correspondingly valuable. [128] On the other hand, the Zhang and Kecioglu method considers the suspended items and the MLE for the data. It is usually applied if the Dixon and Mood method is not indicated when the fatigue limit is not normal, the stress increments are not identical, and the stress increment is greater than twice the standard deviation. [126].

Wiscott and coworkers [117] applied the staircase method to compare 4 abutment types to determine whether the connector's antirotational mechanism participates in fatigue resistance. The goal of the experiment was to determine the stress level at which 50% of the specimens would survive 10^6 cycles and 50% would fail. In this study, the specimens were loaded to their long axis for a maximum of 10^6 cycles. After this period, the test was halted and the specimen was examined to determine whether it was broken or intact. If it had survived 10^6 cycles, the specimen was said to have "run out" and the next specimen was loaded to the previous magnitude plus 5N. The same force (5N) was subtracted from the former load magnitude if the previous specimen had failed. At the end, 30 samples were tested in sequence.

Although this mechanical fatigue testing method is an easy way to estimate the fatigue strength of a component/material considering only "up and down" sequential technique, its accuracy and many sensitivity factors should be questioned, which limits its indication.

Considering such limitations, the test is not conducted in a wide force range and the sample is not submitted to extreme stress values, which could mask the maximum strength of the material. In addition, the number of cycles to determine the failure or survival of each specimen is predetermined, avoiding the simulation of material performance for a longer period of time. Thus, all these limitations should be considered by the researcher to indicate the staircase method as a reliable testing alternative for fatigue strength of materials.

6.3.5. Step-Stress Accelerated Life-Testing

The step-stress accelerated life-testing (SSALT) method consists on a mechanical (fatigue) test for shortening the life of materials or hastening the degradation of their performance. The aim of such testing is to quickly obtain data which, properly modeled and analyzed, yield desired information on product life or performance under normal use. [129] The SSALT method allows the prediction with confidence intervals (based on calculation of a master Weibull distribution) of the life expectancy of a given material under specified loading. For the mathematical approach, computer software is available for life expectancy calculations.

In the SSALT, a specimen is subjected to successively higher level of stress. First, each specimen is submitted to constant stress during a predetermined length of time. The stress on this specimen is thus increased step by step until its failure. Usually, all specimens go through the same pattern of stress levels and test time. [129] SSALT has been widely applied for metals, plastics, dielectrics and insulations, ceramics, rubber and elastics, food and drugs, lubricants, protective coating and paints, concrete and cement, building materials, and nuclear reactor materials. Similar process has been also observed for biomaterials used in dentistry; such as ceramic restorations, dental implants and adhesive bond material. [110,112-114,125,130-133].

Previous work [110,112-114,130,134-141] using SSALT in dentistry, the (SLF) is the first step applied prior to this method. Based upon the mean load to failure from SLF, step-stress accelerated life-testing profiles (usually 3 profiles defined as mild, moderate, and aggressive) are determined. The rationale for utilizing at least three profiles for this type of testing is based on the need to distribute failure across different step loads and allows better prediction statistics, narrowing confidence intervals. [134] Mild, moderate or aggressive profiles refer to the increasingly step-wise rapidness in which a specimen is fatigued to reach a certain level of load, meaning that specimens assigned to a mild profile will be cycled longer to reach the same load of a specimen assigned to either moderate or aggressive profiles. The profiles usually start at a load that was approximately 30% of the mean value of SLF and end at a load that was approximately 60% of the same value.

Several previous investigations [112-114,129] have demonstrated that 18 specimens are enough to obtain statistically significant results based on Weibull analysis. Therefore, three specimens are subjected to SLF and the remaining 18 specimens are assigned to a mild ($n = 9$), moderate ($n = 6$), and aggressive ($n = 3$) fatigue profiles (ratio 3:2:1, respectively) (Figure 6-12). Following the parameters of loading for each predetermined profile, the specimens are fatigued until failure (bending or fracture) or survival (no failure at the end of step-stress profiles), where maximum loads are applied up to a limit previously established based on SLF mean value (N).

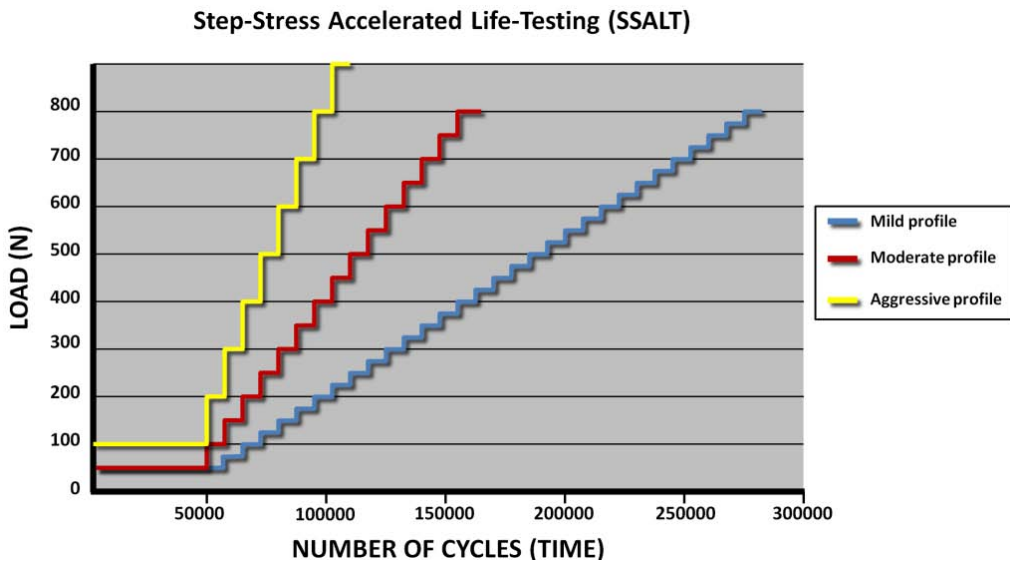


Figure 6.12. Chart illustrating the step-stress (mild, moderate and aggressive) profiles used for an accelerated fatigue testing. Note that the loads started in the neighborhood of 50–100 N ending up to approximately 800–900 N. These values are based upon the mean load to failure from SLF test.

Based upon the step-stress distribution of the failures, the fatigue data are analyzed using a power law relationship for damage accumulation and the use level probability Weibull curves (probability of failure vs. cycles) at a use stress load are determined for life expectancy calculations by using a specific software (Alta Pro 7, Reliasoft, Tucson, AZ). [113] The use level probability Weibull analysis provides a beta (β) value, which describes the failure rate behavior over time ($\beta < 1$: failure rate decreases over time, commonly associated with "early failures" or failures that occur due to egregious flaws; $\beta \sim 1$: failure rate does not vary over time, associated with failures of a random nature; $\beta > 1$: failure rate increases over time, associated with failures related to damage accumulation). Reliability calculation (with two-sided confidence bounds that can be calculated by a variety of methods including the MLE) of tested specimens for completion of a given number of cycles (also named as mission) at a specific load level (Figure 6-13) can also be performed. If the use level probability Weibull calculated beta (β) was less than 1 for any tested group (meaning that the crown-implant system failure is controlled by materials strength rather than damage accumulation from fatigue testing) then a Weibull two-parameter can be calculated (Weibull 7++, Reliasoft, Tucson, Arizona, USA) using only the final load at failure or survival of specimens (disregarding the number of cycles). [142].

An instructive graphical method to determine whether these data sets are significantly different (based upon non-overlap of confidence bounds) is the utilization of a Weibull 2-parameter contour plot (Weibull modulus (m) versus Characteristic Strength ($\eta = \text{Eta}$)) (Figure 6-14). "m" is an indicator of strength reliability and/or the asymmetrical strength distribution as a result of flaws within the material. [143] A higher "m" indicates smaller and/or fewer defects (greater structural reliability), and a lower "m" is evidence of greater variability of the strength, reflecting more flaws in the system, and a decrease in reliability. [143].

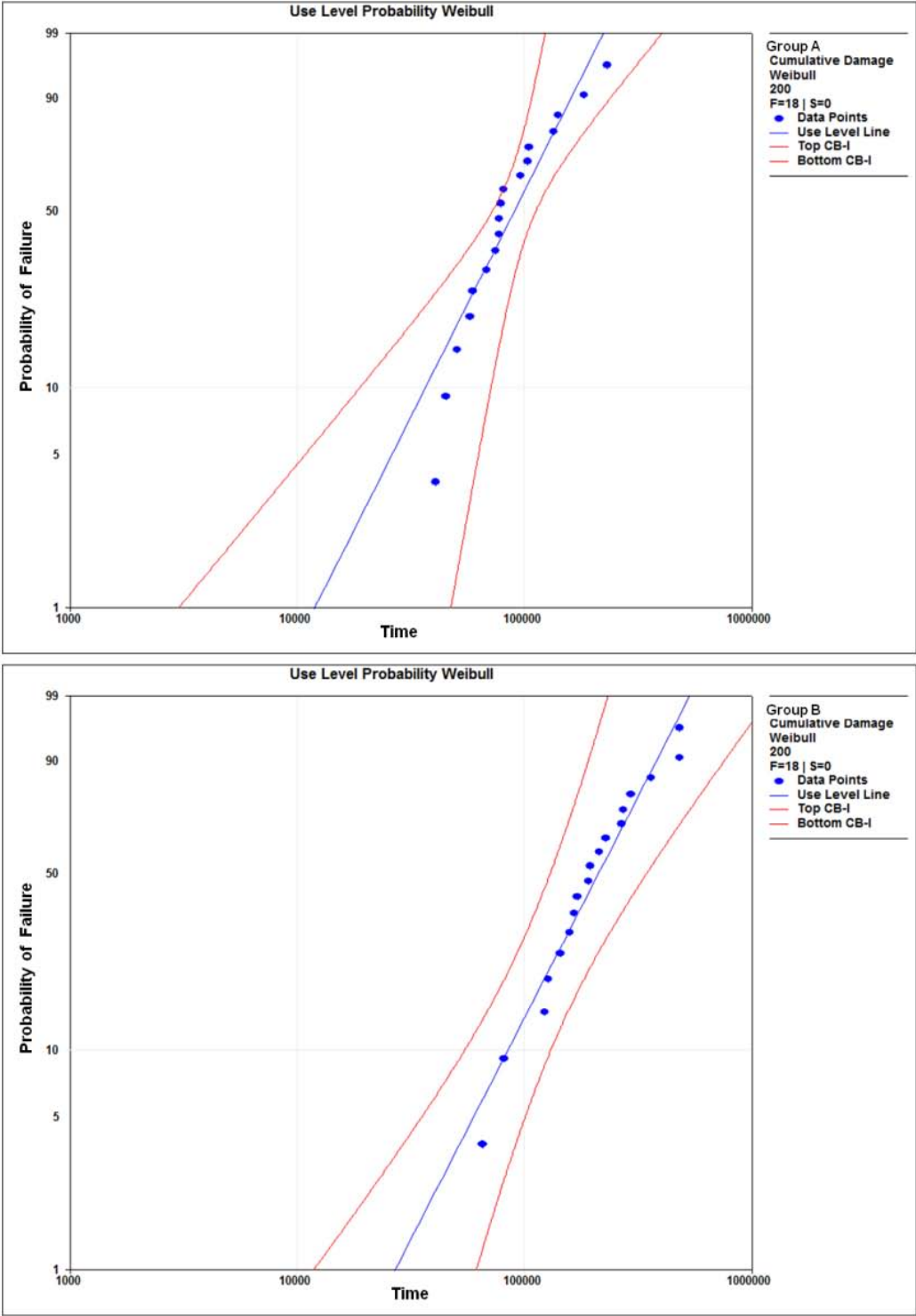


Figure 6-13. Use level probability calculation a hypothesized test showing the probability of failure as a function of number of cycles (time) with a use stress of 200 N It should be assumed that the cumulative damage from loads reaching 200 N might lead to restoration survival in a higher number of specimens in group B than in group A.

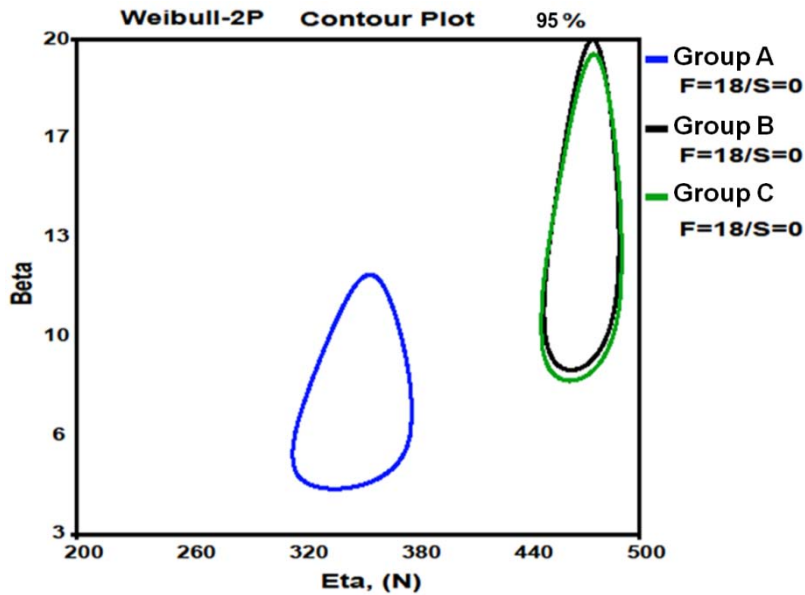


Figure 6.14. Weibull parameter contour plot (Weibull modulus (m) versus Characteristic Strength ($\eta = \text{Eta}$)) for a hypothesized test in which the " m " value and the " η " were significantly higher for groups B and C compared to group A. In addition, the characteristic strength (η), which indicates the load at which 63.2% of the specimens of each group may fail, was also significantly higher ($p < .05$) for groups B and C compared to group A.

In dentistry, the SSALT method can be employed using a servo-all-electric system where the indenter contacts the specimen surface, applies the prescribed load within the step profile and lifts-off the material surface. However, the step-stress method can also be conducted with an electrodynamic testing machine to simulate the mouth-motion fatigue (MMSSALT). [114] Thus, the specimens can be tested in either axial or off-axis loading orientation. [113].

The applicability of SSALT in the evaluation of implant-supported restorations was demonstrated for instance in a study performed by Bonfante and coworkers [109] where the reliability and failure modes of SSALT of implant- Y-TZP versus palladium-silver alloy (PdAg) implant-supported 3-unit bridges were evaluated. In Bonfante's study, in order to mechanically challenge the bridges, the fatigue test was undertaken with all specimens held at a 30-degree axial inclination, with the indenter contacting the pontic lingual slope of the buccal cusp, providing a bending component during loading. The mouth motion fatigue testing (where the indenter slides approximately 0.5 mm along the surface because of the slope presented by specimen angulation and anatomy) was then performed at 2 Hz using an electrodynamic fatigue testing machine.

Similarly, Mitsias and coworkers [111] evaluated the fracture strength and accelerated fatigue reliability of metal and zirconia abutment systems using the SSALT method. The fatigue step-stress test used the same geometric configuration as described above by Bonfante and coworkers. [109] Some alterations in the SSALT can be suggested by other studies as a different distribution ratio of specimens in the fatigue profiles for 4:2:1 [112,114] and placement of an aluminum foil with a thickness of 0.5mm between specimen and indenter to evenly distribute the load, therefore minimizing chances of surface damage where the load was applied. [110].

The main advantage of a step-stress test is that it quickly yields failures. The increasing stress levels ensure this. However, quick failures do not guarantee more accurate estimates. A constant fatigue test with a few specimen failures usually yields greater accuracy than a shorter step-stress test where all specimens fail; i.e., the total time on test (summed over all specimens) determines accuracy, but not the number of failures. [129] On the other hand, one disadvantage of step-stress tests is that under clinical conditions most specimens run at constant stress, not step stress. Thus, the tested model must properly take into account the cumulative effect of exposure at successive stresses. Moreover, the model must also provide an estimate of life under constant stress that should not exceed 3-4 times the average number of cycles employed throughout the test for all groups. [129].

Conclusion

The biomechanical behavior of all components used in implant prosthodontics represents an important factor in determining the life expectancy of the restoration.

Ideally, the *in vitro* tests should be designed in such a way that not only answers a specific question, but also allows a generalization of results that contribute to the discovery of laws or rules relating to fatigue life. However, in practice this is very difficult to be achieved. There are always unknown and uncontrollable factors that produce scattered information, even considering that the tested specimens are identical. However, even presenting some limitations for clinical extrapolation, the *in vitro* methods such as computer simulations and mechanical tests provide applicable information on the mechanical performance of implant-prosthesis assemblies, especially when the association of methods is applied.

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Part IV – Animal Models in Dental Implant Research

Chapter VII

Animal Models for Experimental Surgical Research in Implant Dentistry

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Abstract

Since about 20 years ago, implant dentistry research has accumulated an extensive knowledge on the integration of dental implant by investigating with different animals.

The general objective of this chapter consists of working through a critical analysis of the literature in discerning and proposing a possible method to pave the way for an incremental and synergistic balanced approach, integrating the existing animals of today and experimental models presented in the studies conducted with: mice, rats, rabbits, ovine, swine, dogs and non-human primates. The recruitment of an animal species requires that a special attention is paid to the following elements: ethics, genetics, anatomy and surgical approach.

The pertinence and strength of an animal study pertains to the sustainability of its working hypothesis and to its incorporation inside a prospective animal experimental research program. This implies a rationale founded on existing evidence-based experimental surgical research results and on a methodological approach including studies with different animal and experimental models, allowing discrimination through comparison and complementing each other.

It is expected that the reader of this chapter will concretize his thoughts by the elaboration of a self-reflection sheet, incorporating the specific contributions of each animal model and their complementary aspects.

7.1. Introduction and Definitions

Implant dentistry research relates to a relatively recent but rich history of experiments involving animals. The pioneering phase, which is slowly coming to its own completion,

contributed to accumulate an extensive knowledge by investigations on mice, rats, rabbits, ovine, pigs, dogs and non-human primates. Nowadays, if the animal candidates remain the same, the necessity of conceptual and methodological improvements appears dramatic in order to obtain more information from each study and benefit from a multiplier more than a simple additional effect by collecting results from several studies.

The objective of the present chapter is to attempt an integrative approach of animal models used in order to contribute to replacing a compilation of parallel generated data and observations by a more synergistic-based response to a scientific question.

In experimental surgical research it is critical to outline the originality of this question, leading to a precise working hypothesis as stated in the definition inspired by the Academy of Surgical Research (Eden Prairie, MN, USA):

The experimental surgical research is a part of the surgical research and examines and evaluates in animals innovative and conventional ideas related to surgery through scholarship analysis of data and the generation and investigation of hypothesis.

The stringency of this statement fully applies to dental implant research: the type of surgery performed is often not limited to the insertion of a device into the jaw but includes routinely extensive bone regeneration operations. Therefore, this kind of surgery corresponds to a major procedure that penetrates and exposes a body cavity, produces substantial impairment of physical or physiologic function, or involves extensive tissue dissection. This type of surgical intervention is thus considered a major survival surgery. In this context the choice of the model must be guided by an argued scientific rationale founded on evidence-based experimental surgical research data and a critical analysis of the international literature. Consequently, this choice must incorporate the best knowledge on comparative animal anatomy, physiology and medicine, guiding the animal species selection. An animal model may be defined as a non-human living animal with an inherited naturally acquired or induced pathological process or lesion allowing for the resolution of a research hypothesis and resembling a similar condition in the target human species. [1] It is important that the animal population study presents homogeneity in race, species, gender, age and weight.

Beyond the animal model itself but being part of it, the experimental model is defined as the association of an animal type benefiting from a special surgical procedure which elicits a specific type of defect at a dedicated anatomical site. Consequently, the choice of the model has to be argued by outlining the anatomical and physiological adequacy of the animal with the foreseen surgical procedure and for implant dentistry, with the types of defect or bone bed. Furthermore it is, in our sense, critical that the model accommodates the pre-translational or translational study objectives.

The sequential approach has two phases: pre-translational (investigations on the basic properties required for a dental implant) and translational (investigations on a particular clinical indication related to a specific surgical procedure). This appears mandatory and allows to come closer to human situations and treatments without drawing any definitive direct correlations.

An animal study finds its strength if it can be correlated to other animal trials belonging to the same research program conceived prospectively. This implies a progressive and incremental approach including studies with different animal/experimental models which complement each other and allow for discrimination through comparison.

Since 20 years ago, an impressive knowledge has been accumulated with respect to the osseointegration of dental implants in animals, but each study was designed or is taken for its own.

Three outstanding literature reviews comparing different animal models by Pearce et al., [2] Pellegrini et al. [3] and Bagi et al. [4] inspired the present work which is also founded on our own experience. [5] The general objective of this chapter consists of discerning and proposing a possible method, paving the way for an incremental and synergistic balanced approach that integrates today's existing models based on research conducted in mice, rats, rabbits, ovine, swine, dogs and non-human primates. The specific aim wishes to head to the presentation of a reflection tool expected to be concretely implemented into a self-reflection sheet before starting an animal trial.

7.2. Mouse

The mouse is the most commonly used animal research model with hundreds of established inbred, outbred, and transgenic strains. Most laboratory mice are hybrids of different subspecies, and they come in a variety of coat colors including agouti, black and albino. They are relatively inexpensive, easy to handle and reproduce quickly in laboratory settings. The mouse genome has been sequenced, and many mouse genes have human homologues. Therefore, genetic variants of mice were bred to mimic human diseases such as obesity, osteoporosis or diabetes, and this opens wide research perspectives for implant dentistry. Knock-out mice presenting tailor-made gene failures and related phenotypic outcomes should be part of an integrative strategy of research in guided bone regeneration and implant dentistry.

Beppu et al. tested titanium coated plastic implants in two different genetically altered mice: senescence accelerated prone-mice and senescence resistant-mice. [6] They found differences in bone mineral density with lower levels in the osteoporotic model (senescence accelerated prone-mice).

Nowadays, the main type of modified mouse used in implant dentistry is the nude one. This animal has a deficient immune system with a lack of T cells. This qualifies it as a candidate for a combination of implants with cells of various origins (i.e. human) without developing strong immunological reactions. Xu et al. demonstrated that osterix, a transcription factor, is contributing in the bone marrow stromal cells mediated enhanced osseointegration of implants in this type of mice. [7].

Examples of studies in mice where dental implants were tested intra-orally for tooth replacement do not exist. Furthermore, as reported in the above publications, the field of interest in a mouse treated with implants is in extra-oral bones, such as the tibia, femur and calvaria. For these purposes, dental implants of reduced sizes are inserted (lengths approaching 2 mm and a diameter around 1 mm). Rahal et al. tested the myelointegration of implants into bone marrow. [8] Although hypothesized that B cells were of concern at the implant-bone marrow interface, the authors concluded that implants, when tested in mouse femurs, were well tolerated.

Bone growth around titanium implants was investigated in a murine *calvaria* model by Freilich et al. [9] In this case, the bone formation with bone morphogenetic protein (BMP)

treated implants was successful. The authors claimed that this murine calvarial bone formation model is better suited than the ectopic muscle pouch models otherwise used to show bone regeneration. A study in mouse muscle pouches was successfully completed by Simon et al. to test bone induction on implants supplemented by BMP and placed in this heterotopic site. [10].

7.3. Rat

The main strains used are the Wistar rats or the Sprague-Dawley rats. Wistar rats are variants of inbred albino rat whereas Sprague-Dawley rats are of an outbred origin. Male rats are more adapted to implant dentistry as their body weight (450-520 g) is higher compared to female rats (250-300 g).

The rat is mainly recruited for extra-oral implantation experiments into the calvaria or the tibia and femur since rat jaws and teeth are small. Therefore, only a few studies dealt with intra-oral placed implants, and they described implantations of mini-implants into bones rather than teeth replacement. It is important to emphasize the contribution of Haga et al. who extensively documented the bone formation and maturation processes around implants placed in the maxilla. [11] Rinaldi and Arana-Chavez examined implants in contact with the periodontal ligament of adjacent teeth in the mandible. [12] Hou et al. compared tissue reactions after implantation under loaded and unloaded conditions and concluded to a higher bone-implant contact ratio for the loaded implants group. [13].

Mini-implants were also used by Wen et al. in a study that aimed at establishing an experimental model in addition to investigating the effect of the release of BMP from peri-implant scaffolds or implant surfaces. This new rat model showed to be successful, and the scaffolds supplemented with BMP appeared to be efficient. [14].

The main sites for dental implant testing in rats are in extra-oral bones, such as calvaria, tibia and femur sites. Shimizu-Ishiura et al. used mini-implants in rat femurs. [15] In this study, trabecular bone growth was examined around titanium implants, with or without the adjunction of enamel matrix proteins. A similar experimental setting was implemented to analyze the effects of simvastatin, a cholesterol-lowering drug, in a rat tibia model. [16] Apatite-coated titanium, which incorporated BMP and heparin, was ascertained to have enhanced bone forming abilities, too. [17] Except from studies on growth factors or bone inducing agents, implant materials or surface modifications are also commonly studied in rat extra-oral models. The early biological response to commercial pure titanium, in the form of semi-cylindrical bullets implanted into the femurs of Wistar rats, was addressed in a study by Watanabe et al. [18] Kohal et al. compared titanium and zirconia implants with push-tests and histology. [19] The examination showed that titanium and zirconia, when surface-modified in the same way, showed similar osseointegration properties.

Special strains of rats are foreseen to mimic human diseases such as diabetes (Biobreeding diabetes prone rats) or hypertension (Zucker rat). The osseointegration of dental implants can be tested on genetically modified diabetic rats. Hasegawa et al. tested implants in type 2 diabetic rat femurs. [20] The diabetic rats showed significantly less bone volume formation than the control group, making this study a valuable pre-clinical experiment

involving compromised animals. In another investigation by Siqueira et al., Wistar rats were treated to become diabetic, and insulin was concluded to have a positive role. [21].

In all the above animal trials, a routine radiological assessment set up to control the positioning of the mini-implants belongs to the treatment plan. Aside from X-ray analysis, however, other imaging technologies should also slowly access the operation suites. Micro-computed tomography is routinely used to investigate, on retrieved bone samples, after animal termination. Because of its size, the living rat is a suitable candidate for micro-computed tomography follow-up after an implant placement. [22] Eventually, the time-course micro-computed tomography and the histological analysis can complement each other.

7.4. Rabbit

The rabbit, which represents the animal of choice in about one-third of all musculoskeletal studies, is also commonly used in implant dentistry. [23] It is generally admitted that it corresponds to a pre-translational animal model devoted to the screening of dental implant designs or materials prior to testing in a larger animal which incorporates more translational aspects. In comparison to other species, such as primates, the rabbit has a faster bone turnover with significant intracortical haversian remodelling. [2] However, this is not a disadvantage if rabbit experiments are planned as screening studies intended to verify a hypothesis related to osseointegration features and to separately and comparatively document the components of a more complex construct, such as scaffolds supplemented or not with signalling substances. Among all strains of rabbits, the New Zealand White with a body weight between 2 and 5 kg is the most popular. It also appears in genetically altered specimens like the Watanabe, St. Tomas, and Houston RT. [24] Other breeds are the Dutch, the Polish and the Loop rabbits. In the case of bone surgery, as larger sized animals offer more space for the surgical procedure, the rabbit became the candidate for combined implantations of dental implants and bone substitutes.

Ovariectomized female rabbits are expected to mimic post-menopausal conditions but although the bone mineral density conducted by dual energy X-ray absorptiometry showed a significant loss of bone mass, it was not possible to observe differences in the bone to implant contact measurements between the osteoporotic and healthy animals. [25].

The rabbit offers a variety of anatomical locations as potential candidates for implant placement: calvaria, maxilla, mandible, and knee (femur and/or tibia). If the jaws and knees are large enough to enable the insertion of dental implants manufactured for human purposes, this is not the case in the calvaria where mini-implants are required. Historically regenerative materials such as allografts, xenografts and autogenous or synthetic bone grafts were extensively tested in skull bones by drilling 1 to 4 cylindrical defects since this bone is close to the skin, making for easy surgical access. Furthermore, the calvaria location allows for the assessment of implant stability with the help of transcutaneous measurements. [26] Studies where special implants were used to evaluate osseointegration, together with regenerative materials, revealed that a homogenous frozen bone was equally effective as an autogenous bone in bone remodelling around titanium cylinders, indicating that a frozen homologous bone can possibly replace an autogenous bone in these cases. [27] It seemed that the presence

of stem cells added to titanium implants, improving the bone formation in this experimental model too. [28].

The rabbit maxilla offers the opportunity to compare autogenous bones and xenografts, supplemented or not, by different concentrations of BMP in a sinus floor elevation experimental model. [29, 30] An alveolar ridge augmentation, without a sinus floor elevation, was also examined in the maxilla. Masago et al. confirmed the importance of an appropriate gap between the inert materials to allow new bone progression. [31].

Freilich et al. described a procedure for implant placement into the rabbit mandible. [32] The animal received custom-designed implants and, in some cases, scaffold material in posterior sites. The coronal aspect of each implant was left outside the lateral aspect of the posterior mandibular bone, but covered by periosteum, muscle, subcutaneous tissue, and skin. Bone formation was later quantified inside a region of interest defined between the native bone and the upper end of the implant. The lateral implantation was also of an advantage to position a distraction device parallel to the mandibular body and secure it with self-tapping screws. [33] A sequential activation of the device allowed for a direct force transduction and precise setup of the gap distance to be investigated on. The addition of stem cells to a scaffold filling the gap seemed to contribute to the consolidation of the mandible. [34].

Although the mandible appears suitable for experiments with implants, [35] with bone-grafting materials [36, 37] or with implants surrounded by a scaffold, [32, 38, 39] materials were also evaluated in the knee (femur and/or tibia). This site appeared quite adapted to biomechanical measurements obtained by torque removal combined with histological observations and measurements. Franke et al., when examining the bone forming/osseointegrative properties of enamel matrix proteins around titanium implants in a rabbit tibia/femur model, found that the torque removal method was of value to evaluate the osseointegration. [40] In a recent study by Pak et al., [41] the surface characteristics of implants were tested in a similar model. The bone-to-implant contact and the bone volume density were assessed around three different types of titanium implant surfaces: tricalcium phosphate-coated, anodized, and turned (machined). This study showed that osseointegration occurred in all investigated types of surface-treated implants. Carmagnola et al., [42] described a procedure for grafting/implanting into rabbit tibiae. They drilled two cylindrical defects which were filled with commercial bone graft particles or left untreated, and covered with a collagen membrane. Later, a dental implant was inserted at the center of each previously created defect. The osseointegration seemed to be better when a grafting material was used.

The fact that in the above studies significant statistical differences cannot be shown between grafted and ungrafted defects [42, 43] or between implants emphasizes the importance to implement convergent methods (histology and biomechanics) and the need to investigate this type of experimental model. [41] In the past, Lundgren et al. [44] already met difficulties to discriminate between two types of implants by using only a histomorphometrical analysis. To overcome this difficulty Gottlow et al. and Sennerby et al. proposed a complementary approach based on histology and biomechanics which could be adapted to dental implants. [45, 46] Monjo et al. further developed a surgical method initially established by Ronold and Ellingsen and maximized the experimental data collection after the cortical positioning of disk samples on the tibia by studying the association of pull-out tests with gene expression and volumetric bone mineral density of sub-implant cortical bone obtained by micro-computed tomography. [47] Similar approaches associating convergent

methods should contribute more in the near future to increase our knowledge on the biological performances of material surfaces in the rabbit model.

7.5. Ovine

The number of sheep included into animal trials for implant dentistry research is increasing. Adult sheep or goats possess bone dimensions which correspond to those of humans, simplifying the use of human dental implants.

Sheep bone has a bone density which is 1.5-2 times greater than human bones. [48] However, the age, gender and anatomical site yield different density proportions. [2] Dvorak et al. checked osteoporosis in sheep mandibles, using geriatric sheep submitted to ovariectomy, vitamin D deficiency and hormone therapy. [49] Comparatively to the controls, the bones of these sheep were osteoporotic, indicating the usefulness of this type of animal model. It is speculated that old ewes (9-10 years old) could present bone structures approaching post-menopausal women, making these animals suitable for osteoporosis studies. The age/osteoporosis aspect in combination with different implant surface treatments was tested by Giavaresi et al., who aimed at finding the most suitable implant for all kinds of bones, normal, aged and osteoporotic. [50] The sheep's trabecular bone model appeared to be of value for this purpose.

There have been a few studies on testing different types of implants or implant coatings in sheep intra-oral sites. Bartold et al. compared the effect of different implant shapes and surface designs on bone microdamages and microcracks. [51] Gutwald et al. showed that there was a favorable effect from using BMPs in sinus floor augmentation procedures with immediate implant placement. [52] Jakse et al. tested the influence of laser treatment on the osseointegration of dental implants. [53] Saffarzadeh et al. reported that there was a higher efficacy of macroporous biphasic calcium phosphate fibrin composite grafts compared to autologous bone grafts in sinus lift augmentation procedures. [54] Sauerbier et al. developed a new and minimal invasive method to investigate the bone formation and osseointegration of dental implants in a maxillary sinus. [55] A comparison of bovine bone mineral alone, and in combination with mesenchymal stem cells, was conducted regarding their respective effects in this indication.

Sheep have their specific oral biomechanics inherent to their constant ruminant activity, and this contributes to a high degree of implant failures in alveolar ridge augmentation procedures. Vlamnick et al. examined sheep as a potential animal model for immediate implant placement in fresh extraction sockets. [56] Clinical and histological observations demonstrated a poor implant osseointegration characterized by soft tissue in-growth into the extraction sockets and loss of the materials. However, in another study by Rachmiel et al., alveolar ridge augmentation, through distraction osteogenesis and enhanced bone regeneration through the use of BMP followed by implant placement, was successfully performed. [57].

The goat iliac bone has been evaluated by Schouten et al. as a dental implant test site. [58] Their study proposed a new model for the standardized evaluation of bone responses to implants. Their findings suggested that the iliac crest implantation model is suitable for evaluating the osteogenic response to bone implant materials and represents a deliberate

alternative to the already existing experimental models. A standardized surgical approach allows the comparison of nine different implants per side. The examination of the influence of an implant bed preparation in the pelvises of sheep was done by Stübinger et al., using laser ablation, piezoelectric surgery or drill osteotomy. [59] According to the authors, laser and piezoelectric osteotomy seemed to be at least comparable to drill osteotomy when concerning early osseointegration of implants. A study about surface modifications and materials using the same implant geometry was performed by Langhoff et al. [60] in the iliac bone of sheep and Ferguson et al. tested different implants in sheep pelvises comparing the torque needed to remove them. [61].

The sheep's tibia was found to be a good model for testing different implant types. A study investigated peri-implant osteogenesis in different zirconia sandblasted endosseous titanium surfaces at a short-term after surgery. [62] Histological sections, microhardness measurements, and electron microscopy observations revealed that all the implants were biologically fixed by a newly formed woven bone arranged in thin bone trabeculae. A similar study, comparing different implant sandblasting procedures, was performed by Bacchelli et al. The authors found that a zirconia sandblasting of implant surfaces yielded the best results. [63].

In a goat's femur, Junker et al., testing two different calcium phosphate coating procedures for titanium implants, found that both coating methods were equally effective. [64] In the same model, Nikolidakis et al. concluded that a low dose of Transforming Growth Factor had a negative effect on the osseointegration of implants during the early post-implantation healing phase. [65].

7.6. Swine

The swine is one of the major animal species used for translational purposes in pharmaceutical research. It was recently positioned as a candidate among other species for use in musculo-skeletal surgical investigations. [2] The testing of dental implants requires the use of adult animals that have reached their jaws osseous and dental maturity. This contributes to the disqualification of commercial breeds of domestic swine which, at one year of age, generally weigh more than 100 kg. Consequently, the latter presents researchers with difficulties in handling, housing and administering anaesthesia. Furthermore, these land farm animals are non-barrier animals presenting risks of non-identified latent infections and transmission diseases.

The miniature swine helps to overcome the above issues. Different strains of miniature pig, weighting about 30-40 kg at their age of dental maturity, were developed since the sixties: Yucatan (in its two forms of minipig and micropig), Hanford, Sinclair Hormel (also called Minnesota), Pitman-Moore, Kangaroo Island, Ohmini, Lee Sung, Morini and Göttingen. The minipig, when bred as a barrier animal, demonstrates fully controlled healthy conditions. Furthermore, it scarcely develops gingivitis and periodontitis as plaque does not accumulate around its teeth. Some differences in bone development and growth exist between domestic pigs and minipigs, and also among strains of minipigs. [66] Nonetheless, with respect to general bone anatomy, structure, healing and remodelling after reaching their adulthood, swine bone is considered to be representative of human bone. [67] The literature

describes the swine as having bone remodelling processes similar to humans, comprising both a dense trabecular network and intra-cortical remodelling. [68, 69] Furthermore, porcine shows similarities in bone mineral density and bone mineral concentration to human bones. [70] The movement of the temporomandibular joint and the size, shape and anatomy of the minipig mandible are similar to those of humans. The bone regeneration rate of the adult minipig mandible (1.2-1.5 mm/day) is comparable to that of humans (1.0-1.5 mm/day). [71] In a publication dealing with the bone physiology of the female Göttingen minipigs, standardized data was disclosed on bone growth rate, biomarkers, bone mineral content and density. [72].

Efforts are currently being conducted to develop diabetic type 2 swine in land race pigs and minipigs. [73-77] Obese-diabetic pigs share many similarities with obese-diabetic humans such as delayed wound healing [78] and reduced bone mineralization. [79] These abnormalities should make the obese-diabetic minipig/pig particularly useful for pre-clinical studies in implant dentistry and bone regeneration.

All the above features render the minipig more popular for studies on bone regeneration in areas foreseen to be treated with implants. [80-85] According to Schlegel et al., specimens benefiting of the preparation of monocortical critical size defects in the skull and treated with calcium phosphate materials exhibited no significant difference of the mineralization rate between human and the porcine models. [86, 87].

The sinus model is well established for the comparison of the biologic behavior of bone grafting materials and bone growth factors in implant dentistry. [88-92] The lateral part of the maxilla is exposed through an extraoral incision placed infraorbitally, and the buccal soft tissues are reflected. [88] Fenner et al. examined the sinus height needed for a combined implantation and an inlay augmentation procedure by using a particulated iliac bone graft. [93, 94] The results showed that the combination of maxillary inlay grafting and a simultaneous implant placement did not hinder osseous integration and implant stability even though the alveolar crest was initially reduced. Buser et al. published the pioneering works in using an intra-oral approach to access the maxilla of the minipig. [80, 95] These authors demonstrated the rationale and usefulness of this experimental model for implant dentistry research. Moreover, Buser et al. [96] showed, for the first time, its ability to discriminate between a non-hydrophilic and a hydrophilic titanium surface as to show differences between a biofunctionalized one with a monolayer of polymer, supplemented or not with a RGD sequence. [97]. Gottlow et al. and Elian et al. intra-orally accessed the mandible to evaluate the osseointegration of titanium-zirconium compared to titanium implants and to investigate the behavior of the bone around the implants inserted at an inter-implant distance less than 3 mm. [98, 99] According to Buser et al. and Jensen et al., the lateral portion of the mandible and ramus represent a valuable alternative to the maxilla. [81, 100-102] They successfully compared different bone substitute materials in membrane protected cylindrical bone defects at this site.

7.7. Dog

First introduced in the 60's for research in periodontics, the dog remained popular as a model for periodontal surgery. In this field, studies address both spontaneous and

experimental periodontal diseases in order to understand the etiopathology of this sickness, [103] its semiology, and the mechanisms of periodontal destruction and healing. Within this context, an extensive research aims at finding materials, growth factors and other signalling molecules to treat periodontal bone and soft tissues lesions. This could turn out to be of great importance for implant dentistry research. Clinically, periodontal diseases and the consecutive need of dental implants to replace the missing teeth are closely linked. Thus, it appeared obvious to recruit dogs for dental implants testing.

Based on history and their previously broad use, one of the most preferred strains in implant dentistry is the Beagle dog. However, this animal is highly affected by spontaneous periodontitis. [104] This renders its use questionable for studies focusing on bone regeneration under fully controlled and stable bone conditions. Larger dog breeds, such as greyhounds, mongrel, labradors, German sheepdogs or mixed-breeds are becoming more widely used in dental implant research. The fact that dogs are companion animals should be taken into consideration before recruiting them for an animal trial. Their involvement attracts a rightful public attention, and researchers should develop convincing argumentation and be ready to explain this choice.

It has to be noticed that if the hygienic maintenance procedure is not perfectly set up and implemented these animals will accumulate dental plaque. This apparent drawback makes them suitable for studies on biofilm deposition and occurrences of peri-implantitis. Implantations into the radius or the tibia of dogs present the advantage of easy access and avoid any preparative phase of surgery in the form of teeth extractions. This strategy seemed to be of value to investigate, with flexibility, during early times of implantation. [105, 106]

Different dental implant shapes, surfaces or coatings were investigated in the alveolar ridge of dogs. [107, 108] As for the insertion into the limbs, alveolar ridge augmentation requires that standardized defects be prepared in a reproducible way and also that there is a reproducibility in histometric data acquisition. [109] As implant success is likely to be dependent on the type and size of the bone defect, Stavropoulos and Wikesjö created narrow/shallow and wide/deep bone wounds and followed their healing in an implant experimental model. [110] Schwarz et al. also underscored the importance and the quality of the bone defect preparation to compare between chemically modified implant surfaces in non-submerged or submerged conditions. [111, 112] The use of BMPs to induce bone inductive processes and, thereby, increase the alveolar ridge to make implantation possible has been investigated by Jovanovic et al. [113] Wikesjö et al. reported that BMPs showed favorable effects in osseointegration and bone formation, but this process can be quantitatively impaired if high concentrations of growth factor are used. [114, 115].

The use of bone grafts constitutes a useful tool to increase the alveolar ridge volume. Araujo et al. demonstrated that autologous bone grafts or bovine bone graft material supported the osseointegration of dental implants. [116].

In dogs, the loss of the alveolar ridge after a tooth extraction and implant placement is a critical issue that has been addressed in many ways. Araujo et al. have examined the alveolar ridge resorption after implantation. [117, 118] They focused on the placement of dental implants into extraction sockets immediately after a tooth removal and found that a bone remodelling process is initiated immediately after extraction and that this might interfere with the osseointegration of new implants. Botticelli et al. came to similar conclusions by looking at the placement of implants into sockets of freshly removed teeth compared to sockets of teeth removed three months earlier. [119] In a later study, Araujo et al. tested the hypothesis

that the tooth extraction method, with or without flap elevation, might influence the resorption of the alveolar ridge. [120] Implants and flap surgery have also been studied to examine the osseointegration of implants when flap/flapless techniques were used to extract teeth prior to implantation. [121] These authors did not find any significant differences between the two methods in the preservation of the alveolar ridge.

Due to its ability to accumulate biofilm, the establishment of peri-implantitis under controlled conditions should be more developed in the dog for research purposes. [122-124] This should lead to a better understanding of peri-implantitis occurrence and follow-up. The peri-implantitis induction methodology and the reported progression rates differ among research groups with respect to the duration of the soft diet [125, 126] and the use of ligatures. [127-129] It can be stated that there is a lot of variability and heterogeneity among experimental models that pretend to study peri-implantitis, and the establishment of standardized procedures is mandatory and urgent. Already accomplished advances in understanding the behavior of soft tissues around implants should be acknowledged and new initiatives encouraged in dog models, and likely in minipigs and non-human primates. [130, 131].

7.8. Non-Human Primate

The non-human primate instinctively represents the most appropriate animal model because of its anatomical, morphological and physiological features resembling those of humans. However, clear ethical considerations arise in using these species for surgical research purposes. The respect of international regulations intended to prevent any trafficking, the risks of zoonotic diseases, the costs and the demanding handling render its recruitment for trials very difficult. [2, 132] Moreover, even if the above requirements are fulfilled, it remains challenging to obtain enough animals to reach the statistical power for a study.

The different monkey and ape strains (marmoset, macaque, cynomolgus macaque, squirrel monkey, baboon, chimpanzee) offer a wide range of sizes and weights: from about 300 to 350 grams for certain marmosets, to sizes similar to human teenagers for adult chimpanzees.

Non-human primates possess both deciduous and permanent dentitions with a morphology of teeth and roots which is close to that of humans with the notable exception of the canines. Furthermore, the oral healing characteristics of non-human primates closely resemble those of humans, making this animal model exceptionally adequate in implant dentistry research. Histologically, the structure of the periodontium is similar to that observed in humans. These animals form microbial plaque and calculus, but they rarely exhibit a progression of gingival inflammation to periodontal diseases. [3] The baboon is an exception, showing some naturally occurring susceptibility to periodontitis that increased in severity with age. [133].

Non-human primates were involved within a noticeable amount of jaw studies. The early bone formation around two different implant surfaces was compared by Mangano et al. [134] Watzak et al. showed the difference in integration between screw-type and cylindrical-type implant models in a baboon model, demonstrating an improved integration for screw-type

implants. [135] Fögl et al. examined calcium phosphate coatings of implants in long term studies, showing that the coating did not hinder long term osseointegration. [136] In another long term study, baboons received implants but no oral hygiene treatment. [137] Healing time was examined by Vernino et al., showing that no difference could be detected in an experimental model of implant integration a few months after implant placement. [138] A titanium porous oxide was tested in a cynomolgous monkey model by Huang et al. with great success, clearly demonstrating the osteoconductivity of the material. [139] In another study, Kohal et al. showed that zirconia exhibited the same level of osseointegration and showed the same peri-implant soft tissue dimension as the positive control. [140].

Gray et al. demonstrated that clinical methods had the same reliability in the crestal bone level evaluation as histological observations. [141] This kind of creative initiative opens perspectives to avoid future sacrifice of the animals.

Implant loading is likely to be the most important area using non-human primate models in implant dentistry. Implants can be inserted directly after a tooth extraction, or a time period could extend between the two procedures. The immediate loading of dental implants inserted into fresh extraction sites has recently been proposed as a novel approach and has been compared to later implant loading. Mangano et al. examined this in a baboon model. [142] The bone-to-implant contact percentage in the submerged and immediate loaded implants appeared to be similar. Another study, showing that longer waiting periods between extraction and implant loading is beneficial for implant integration was performed by Carr et al. [143] A more ambivalent result in this field was obtained by Suzuki et al. [144] They found that the bone remodelling is different at loaded and unloaded implant-bone interfaces. The nature of newly formed bone varies depending on implant loading times, claimed Romanos et al., who examined three different loading procedures: immediate, delayed and unloaded. [145] Quaranta et al. used macaques to test immediate implant loading and its consequences on the peri-implant mucosa. [146] A general trend in the loading-time field seems to be that the most recent studies using novel implants achieved better results with immediate loading.

In non-human primates, studies that addressed a loss of the alveolar ridge are rare, suggesting that other animal models are more suitable to assess alveolar ridge issues. Miranda et al. addressed the evaluation of alveolar ridge augmentation following the surgical use of BMP. [147] Distraction osteogenesis was examined by Boyne and Herford who reported on a complete osseous regeneration of the nasal floor and alveolar ridge. [148].

Conclusion

Sah and Ratcliffe [149] wrote that there is a substantial need for improved and standardized animal models for tissue engineering and regenerative medicine associated with the musculoskeletal system, and animal models, especially large animal models, are critical to the preclinical step of translating research from bench to bedside. We consider that the same is true for implant dentistry.

Much remains to be done in finding the place of each animal model in a synergistic incremental approach towards a translation to humans. Furthermore, for each animal model,

the incorporated surgical experimental models need to be investigated and fully described, listing their advantages and inconveniences.

The present chapter represents a modest contribution to these efforts. Nevertheless, we pretend that we proposed some background for the elaboration of a self-reflection sheet which should consider the following general aspects: ethics, genetics, anatomy and surgical approach.

Adding the more specific contributions of each animal model, today's state of the art indicates:

- genetic altered mice are suitable for prediction models of implants used in patients with certain diseases, such as diabetes or osteoporosis, and this aspect of mice studies should be more developed in the future;
- the rat, healthy or compromised, represents a screening model, delivering data by help of histology and imaging technologies;
- the rabbit, as a screening model, too, allows to combine histology and biomechanical testing;
- the ovine works as a strong comparative experimental model due to the possibility to insert a large number of implants in its pelvis;
- the minipig is the ideal model for bone regeneration studies associated with dental implants when placed in intra-oral situations;
- the dog should be recruited for studies that address alveolar socket treatment or conducted under compromised oral conditions;
- the non-human primate, particularly the baboon, is a confirmation model which should be reserved to studies on bone loading through dental implants.

In general terms, more attention has to be paid to compromised animal and experimental models which likely will become mandatory in the near future to discriminate between different sophisticated types of implants used in challenging surgical indications. It is expected that each of the above animal/experimental models finds its respective place and contributes to a better incremental integrative approach in implant dentistry research in the near future.

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Chapter VIII

Evaluation of Implant Osseointegration in Small Laboratory Animals

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Abstract

Small laboratory animals are invaluable models for many areas of medical research. The rat and rabbit models have been used extensively for preclinical evaluation of biocompatibility of biomaterials and medical devices used in dentistry. This chapter provides an overview comparison of the strengths and limitations of the commonly available small laboratory animal models frequently used in implant dentistry and related research, explores the advantages and disadvantages of the rat maxilla as a dental implant model, and includes a description of methodologies that may be used to prepare this model in both loaded and unloaded conditions.

8.1. Introduction

Most of our basic knowledge of implant osseointegration has been derived from initial studies of mechanisms in animal models. The animal models that have been used range from implants placed in the maxilla, mandible, tibia, femur, humerus or calvarial bones in small laboratory species (mice, rats and rabbits) and large species (miniature pigs, canines, goats and monkeys). [1] These models have contributed greatly to our present understanding of the osseointegration mechanism and the development of new products and applications in

implant dentistry. Animal testing plays a major role in assessing biocompatibility, osteogenic and osseointegration properties, tissue regeneration and other applications. This research using animal models thus bridges the gap between *in vitro* studies and human clinical studies. However, there is no absolute assurance that the animal tests accurately predict human responses and each research project must give careful consideration to the choice of the specific model. The placement of dental implants in large animals is useful for modeling and testing human implants. The limitations imposed by managing large numbers of large animals have, however, led to the development of small laboratory animal implant models involving titanium implants in both the long bones and jawbones.

This chapter is intended mainly to provide an overview comparison of the strengths and limitations of the commonly available small laboratory animal models involving rats and rabbits, for example, that are frequently used in implant dentistry and related research. In addition, this chapter explores the pros and cons of the rat maxilla as a dental implant model and includes a description of methodologies that may be used to prepare this model in both loaded and unloaded conditions.

8.2. Rodent Model

Rodents, such as mice, hamsters and rats, belong to the cohort of Glires and play an important role in biology and medicine. Since the mouse and rat share many biological characteristics with humans, they are commonly used as model organisms for understanding disease processes and testing treatments. Rodents have been used widely for biomedical research because of specific advantages such as small size, low cost, known age and genetic background, as well as ease of handling and housing. [2] Moreover, it is relatively easy experimentally to manipulate the genetic composition of mice and rats and, thereby, to model human genetic disorders in these animals.

Not all rodents are useful for the purposes of implant and osseointegration research and the limitations of the models selected, as well as the methodology involved, must always be kept in mind. Although mice and hamsters are commonly used species in medical research, they will not be discussed here, due to the limitations of their size which limit their usefulness for testing implants. Other rodents such as the rat are therefore preferred and used whenever possible.

8.2.1. Limitations of the Rat Model

8.2.1.1. Rat vs. Human Bone

The first consideration is the differences between cortical bone in the rat and cortical bone in humans. In human beings, intracortical remodeling removes compact or cortical bone from areas that have experienced microdamage. [3-5] This process involves osteoblasts and osteoclasts in Haversian remodeling. However, under normal loading conditions, the rat cortical bone has no osteons. [6] This difference between rat and human cortical bone suggests that the rodent might be dismissed as a poor model for studies modeling human bone. [6] For this reason, any information relating to the osseointegration of titanium

appliances in the rat must consider the fact that the neck region of the implant, as it relates to cortical resorption, cannot be expected to model that of humans with a great deal of precision.

A second consideration is the rate of bone turnover in the rat which is several times higher than in humans. This difference suggests that extrapolation of the remodeling events from rats to humans may also be misleading. The implication that these architectural and temporal differences undermine the usefulness of the rat model is, however, incorrect. For more than a dozen years, it has been known that a key element in the induction of bone remodeling is the mechanical strain placed on the osteocyte during loading of the bone. [7,8] Regardless of the presence or absence of osteons, cortical bone of both the rat and humans will perceive mechanical strain through the osteocyte and respond accordingly. For this reason, most of the same processes at the cellular and molecular level that are found in humans can be usefully modeled in the rat, even if they are temporally accelerated. [9] Only a very few studies have, however, suggested that the tissue responses to implants can be successfully studied in the rat maxilla. [10-13].

8.2.1.2. Rat vs. Human Implants

Because of limited bone and the small amount of space in the mouth of rats, any implanted device must be as small as possible. For this reason, titanium miniscrews in the range of 1-2mm in diameter and 2-4mm in length have been used to model human dental implants. These miniscrews are designed for orthodontic anchorage, orbital reconstructions or plate fixation on the maxillofacial bones in humans. While the size of these appliances, relative to the size of the oral cavity of the rat, may be almost proportional to the relative size of a dental implant in humans, they are not similar in most other respects. It is well established that the thread design, shape, surface texture and applied torque are all key elements affecting the success of dental implants placed in humans. [14,15] In the case of miniscrews, these factors are essentially ignored. As a result, all differences between experimental groups for each study can only be considered in the context of comparison with other similarly treated rats and not in comparison with human implants. For example, a decrease in the rate of failure in the rat may be a positive outcome in some cases, even if all the implants (control and experimental) in a particular study ultimately fail.

Recent studies by De Smet et al. [16] and others [13] have used specially designed appliances with features that more closely resemble human dental implants. These implants have a thread design and surface treatment which closely match the thread designs and treatments used for commercial dental implants. It is expected that, as more of these studies appear in the literature, the gap between what is observed in the rat model and what is seen in humans will narrow.

8.2.2. Advantages of the Rat Model

The above discussion presents several disadvantages to using the rat as a model for human implants. Large animal models, involving the dog and the pig, for example, have been used for decades as implant models and have the size advantage that allows them to receive full-size human implants. [17-19] The usefulness of large animal models for testing various implant surface treatments and thread designs is immediately apparent. However, there are certain limitations to these models that the rat can help to address.

To reach statistical significance in a comparison of implant failure under one experimental condition along with a control may require ten or more animals per group. If the study is a time course of, say, 4, 8 and 12 weeks, a well-designed experiment will contain one control group and one experimental group per time point, requiring a minimum of 60 animals and 60 human dental implants. To circumvent the large number of animals required, multiple implants are often placed in each animal. From a scientific point of view, a dog with four implants is an “n” of one not an “n” of four. [12].

The rat model is particularly helpful in this respect. With the never-ending need carefully to manage research funds, miniscrews and small, customized implants for anchorage are much less expensive than human dental implants. Large groups of rats are also much more affordable to house than groups of large animals. Because of this, true significant differences between treatment groups can be detected and more experimental treatment groups are possible with the rat model than with larger animals. Another advantage to the rat is the availability of rat models of obesity, hypertension, diabetes and many other disorders typically found in the dental patient. The rodent model is the most commonly used animal model for osteoporosis studies. [20] The ovariectomy rat exhibits most of the characteristics of human postmenopausal osteoporosis. Treatment with smaller amounts of experimental medication may also be an advantage. For these reasons, investigations appear in the literature using the placement of implanted materials in the rat femur and tibia for studying osseointegration. [21,22] A study using the rat maxilla to study the osseointegration of implants, however, first appeared in 1998. [23] Since that time, only a few studies have used the rat oral implant model. [12,13,14,24] The early success of the rat oral implant model suggests that it will continue to have a place in implant research. As previously suggested, design of rat-proportioned true implants with thread designs, surface treatments and other characteristics that more closely model human implants should be encouraged.

8.3. Rabbit Model

Rabbits are small mammals in the family of Leporidae and the order Lagomorpha. The rabbit is a standard laboratory animal in biomedical research and is used as models for a variety of human diseases both genetic and acquired. The rabbit is phylogenetically closer to primates than to rodents [25] and is larger than the rat. For these reasons, the rabbit is one of the most commonly used animals for biomedical research, as it is used in approximately 35% of musculoskeletal research studies. [26] New Zealand White rabbits are often selected because of their short developmental period and their faster bone turnover than primates [27].

8.3.1. Limitation of the Rabbit Model

Rabbits are fragile, very sensitive creatures. They stress very easily during anesthetic and surgical procedures, which might lead to the death of the animal during the experiment. Further, rabbits are larger than rats, making them more expensive to purchase and keep in facilities (Table 8-1).

Table 8.1. Advantages and disadvantages of small laboratory animal models (rats, rabbits)

	Rats	Rabbits
<i>Advantages</i>	Cost effective Ease of handling and housing Large number of animals per experiment Known genetic background	Larger than the rat Short developmental period Large number of animals per experiment Relatively large implant diameter ($\leq 3.75\text{mm}$)
<i>Disadvantages</i>	Small size of animal Small implant diameter ($\leq 2\text{mm}$) Different anatomic structures compared with humans High rate of bone turnover Lack of Haversian remodeling activity Very high self-regenerative bone healing potential	Fragile, very sensitive creatures Stress very easily during anesthetic and surgical procedures Oral cavity impossible to access Different anatomic structures compared with humans Very high self-regenerative bone healing potential

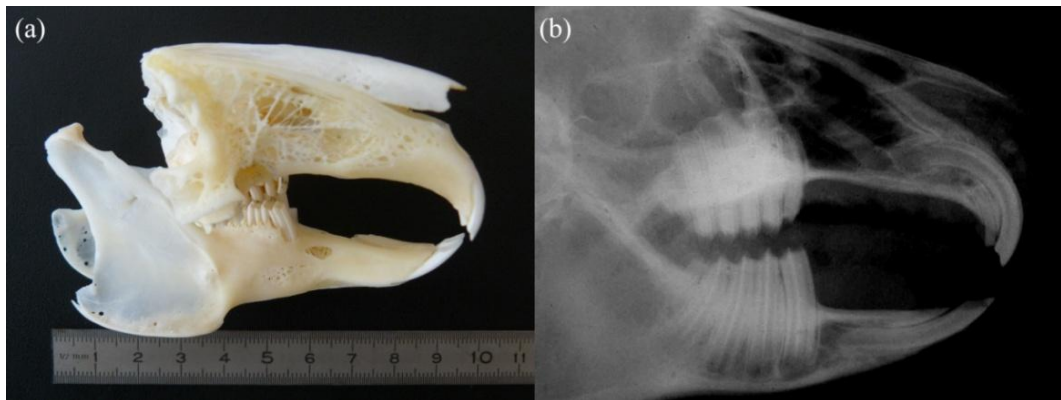


Figure 8.1. (a) Lateral view of the skull and lower jaw of the rabbit. (b) Lateral radiograph of rabbit anterior and posterior teeth.

Another drawback of the rabbit as an animal model for the assessment of multiple implant materials is its size, as it is too small to evaluate commercially available dental implants that are ultimately designed for use in humans. Customized titanium implants were used instead of original dental implants in the rabbit model. However, according to the International Standard, the size of the implants is recommended to be no larger than 2mm in diameter and 6mm in length; again, this is half the size recommended for the other larger species mentioned. [28].

The main difficulty when it comes to oral implantation in the rabbit is that of access. For this reason, oral operations on rabbits are particularly very difficult from this point of view, especially in the molar area of the rabbit which is located deep inside the mouth. Further, the roots of rabbit teeth are very long (more than twice the length of the visible part of the tooth) and the teeth are open rooted, which makes them very difficult to extract (Figure 8-1).

To the authors' knowledge, the intra-oral approach to the rabbit has been used rarely for studies of jawbone healing in natural edentulous areas between the incisors and first molars in the rabbit maxilla and it has been suggested as a useful model for guided bone regeneration research. [29].

8.3.2. Advantages of the Rabbit Model

The rabbit model appears to be more popular when it comes to studying the biological reactions occurring at the tissue-implant interface because of ease of handling, size, the large number of animals that can be used in each experiment and their short developmental period. [27] Unlike other mammals, such as rodents and guinea pigs, rabbits reach skeletal maturity shortly after complete sexual development at approximately 6 months of age [27] and they display significant intracortical remodeling. [30].

Approximately 70-80% of the rabbit's skeleton is composed of compact bone, which is found mainly in the shafts of long bones and the surfaces of flat bones. According to Wang and co-workers, rabbit bone has some similarities in terms of bone composition, bone mineral density (BMD) and fracture toughness to human bone. [31].

The rabbit model has been used extensively for biomechanical studies of dental implants. Different models and tests, including the pull-out test [32] and push-out test, [33] have been used to study the evolution of bone-implant interface strength of transcortically placed implants in both the tibia and femur. The transcortical model facilitates a close fit between the implant and the host bone and simplifies the interpretation of push-out or pull-out tests. Other investigators have used the pull-out test [34] and push-out test [35] in an intramedullary model to evaluate the healing of a gap between implant and bone. However, this model reflects the complex environment into which orthopedic implants are placed rather than the dental implant situation.

8.4. Implant Design

Different implant designs have been used to study osseointegration in different *in vivo* models. Common implant designs used in small laboratory animal models are either screw type (threaded) or cylindrical (rod shaped) and, less commonly, coin, disc, plate or irregularly shaped. The screw-shaped implant (Figure 8-2a) has the advantage of producing good initial stability, whereas cylindrical implants are dependent on exact fit in order to be stable within the bone and produce accurate results regarding their effect on bone integration. [36] Limitations of these implant designs include the mechanically unstable micromovement of the cylindrical implant and the high shear and tensile stress towards the bone tissue during installation of the screw-shaped implant which might affect the tissue response and also partly destroy the implant surface or coating. For this reason, a novel implant design was recently developed by our research group at the University of Gothenburg (unpublished data). Threads were used in the upper part of the implant to provide good primary implant stability in direct contact with cortical bone, whereas the lower cylindrical part of the implant allowed the investigation of *de novo* bone formation within a well-defined healing compartment (Figure

8-2b). In this new design, the implant is in contact with cortical bone, cancellous bone and bone marrow.

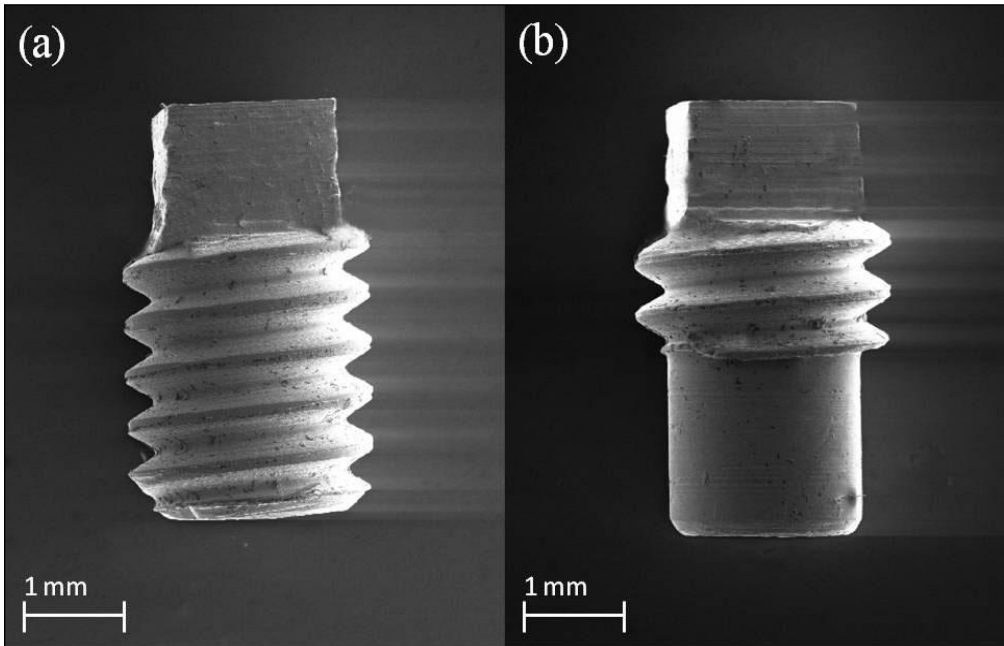


Figure 8-2. SEM photomicrograph of customized titanium rat implants. (a) Screw-threaded implant design. (b) Screw-cylinder implant design.

The advantage of the novel implant design is its close resemblance to the clinical condition due to the presence of the screws in the upper part of the implant, which provide the primary stability for the implant, and the opportunity systematically to modify the portion of the lower part where, at least during the early period of healing, *de novo* bone formation is predominant.

The selection of experimental implant design is dependent on the techniques that are used in the experiment. For example, cylindrical (rod-shaped) implants have been commonly used for pull- and push-out tests, [32,37] screw type (threaded) implants for the removal torque test [38] and coin-shaped implants for the tensile test. [39].

In the rat model, the maximum implant number that should be placed should not exceed 4 implants per animal, for example, 2 in each tibia. However, according to International Standard recommendations, the maximum implant number that should be used per rabbit for the biological evaluation of medical devices should not exceed 6 implants. This is half the maximum number of implants recommended for the other larger species mentioned. [27] It is a standard protocol for the control implants to be included in the study design. These control implants should be made of a material that is well documented experimentally and clinically, such as a machined titanium implant. Further, the implant size for rabbits that is recommended by International Standards should be no larger than 2mm in diameter and 6mm in length. [28] However, many studies have used implants with a diameter of 3.75mm, when the number of implants is reduced to 4 per rabbit. [40,41].

8.5. Anatomic Implantation Models

8.5.1. Long-Bone Implantation Model

Transcortical implantation in long bone in small laboratory models has been widely used in investigations of the early events in the osseointegration process and it adds further information to data that were previously collected from the large animal models. Despite some limitations, this non-functional loaded transcortical model has been widely used and can be regarded as a standard for testing the biological response to an alloplastic material. [42,43] This model can be used for a series of relevant tests to quantify bone tissue response and, in biomechanical tests, to assess the functionality of the newly formed bone at the implant interface. It can be used directly to compare the bone healing capacity of four different biomaterials. Three “test” materials and one “control”, usually machined titanium, were used. In the long bone model, implants are commonly inserted in the epiphyseal part of long bones (cancellous bone), or the diaphysis (cortical bone and medullar cavity) of long bones, such as the tibia or the femur. In the case of diaphysis, the implant is in contact with cortical bone, cancellous bone and bone marrow. Bone formation around the titanium implants in the cortical compartment was described as the simple remodeling of the pre-existing bone. [43] Bone formation around the titanium implant in the medullary compartment was reported as a trabecular repair process or as *de novo* bone formation occurring after the differentiation of new osteoblasts from bone marrow undifferentiated mesenchymal cells. [44]

Animals require an acclimatization period of approximately 14 days after arriving at a new housing facility. The surgical area should be conducive to aseptic surgery. In principle, the surgical area can be located within the housing facilities, thereby limiting stress and potential health hazards to the animals. Surgical procedures should be conducted in compliance with ethical principles for animal research, as approved by institutional guidelines.

In the rat model, both inhalation and injectable anesthetics can be used for anesthesia. Each rat should receive an analgesic subcutaneously postoperatively and the following two days, twice daily. After shaving and cleaning, the medial aspect of the proximal tibial metaphysis is exposed through an anteromedial skin incision. Incision with a 2-cm-length is made through the skin and the fasciae in the tibial metaphysis to expose the tibial crest, followed by skin and periosteum reflection with a blunt instrument.

Two holes are prepared in each metaphysis (proximally and distally) using subsequent enlarging (Ø1.4- and Ø1.8-mm burs) under profuse saline irrigation (Figure 8-3). The holes are irrigated to remove the bone fragments and debris. Implants are installed using a predesigned placement schedule to ensure maximum rotation for the different implant surfaces and placements.

Closure of the subcutaneous layer of the wound should be completed using an absorbable suture material. A nonabsorbable suture material should be used for skin closure. The techniques used in sutural closure play a significant role in wound security. The selection of closure technique depends on mechanical and anatomical considerations, and experimental duration. For example, simple interrupted suture commonly used for short-term studies (<3 days). In contrast, internal running suture used for longer studies (> 6 days).

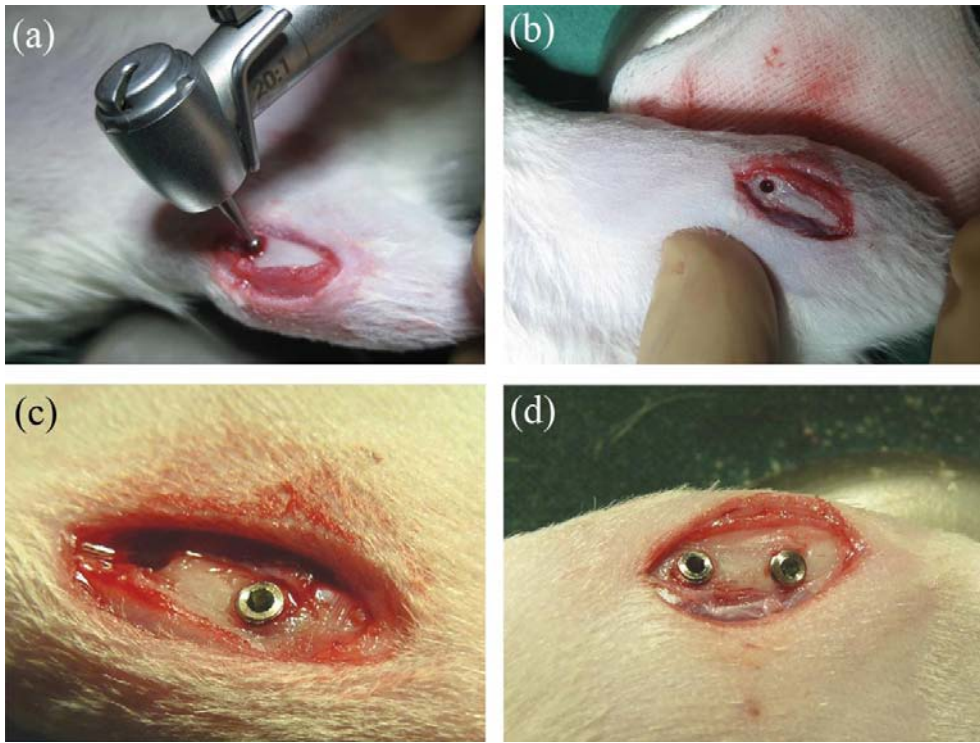


Figure 8-3. A photograph of the basic surgical procedure of implant placement in the rat long bone model. (a) Initial drilling of the tibial bone. (b) Hole enlargement. (c, d) Implant insertion.

After surgery, animals are housed in groups and allowed free postoperative movement, with food and water *ad libitum*.

In the rabbit model, each animal can receive up to three implants in each leg (Figure 8-4): one implant can be placed in the distal femur (mainly trabecular bone tissue), whereas two implants can be inserted in the proximal tibia (mainly cortical bone tissue). The animals are anesthetized prior to surgery with intramuscular injections. The legs are shaved and disinfected with chlorohexidine and the surgery should be performed under sterile conditions. The tibial metaphysis is exposed by a skin incision and blunt dissection of the underlying tissues. Two holes in each metaphysis (proximally and distally) are prepared using a dental guide drill and sequentially enlarged with a 2mm twist drill, a pilot drill and a 3.15mm twist drill during irrigation with saline. Finally, the holes are tapered with a screw tap ($\varnothing$ 3.75mm). The implants are installed according to a randomization schedule. Prior to suturing, cover screws are usually installed on the implants to avoid bone ingrowth. The area is rinsed with saline and sutured in separate layers with resorbable and non-resorbable sutures. The animals are housed separately for 7 days post-operatively. Group housing can be employed during the remaining observation period.

The retrieval procedures are different and are performed to match the intended analysis. For example, for histology and SEM analysis of the bone-implant interface, implants with the surrounding bone are dissected en bloc using a dental disc. On the other hand, for qPCR analysis, a new protocol was established by Omar et al from the University of Gothenburg [45] to evaluate the gene expression of bone at the implant surface and distant to the implant.

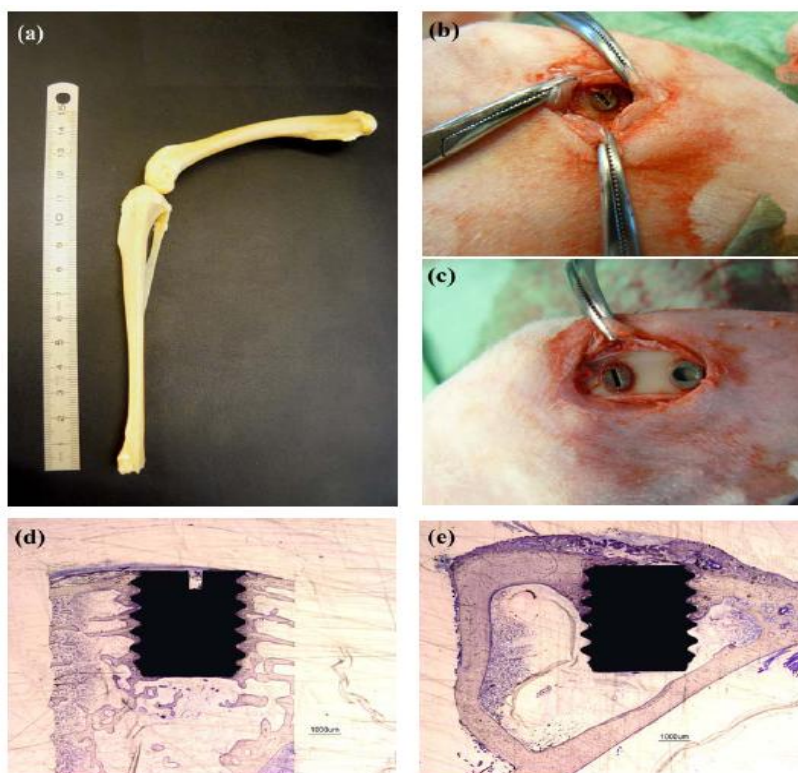


Figure 8.4. A photograph of the rabbit model in which 3 custom-made cylindrical titanium implants were used. (a) Viewed from the side, shows the upper bone, the femur and the lower bone, the tibia. (b) One implant installed in the distal femur. (c) Two implants installed in the proximal tibia. Photomicrographs of undecalcified sections, demonstrating (c) Implant installed in the epiphyseal of femur (cancellous bone), and (d) Implant installed in the diaphysis of tibia (cortical bone and medullar cavity).

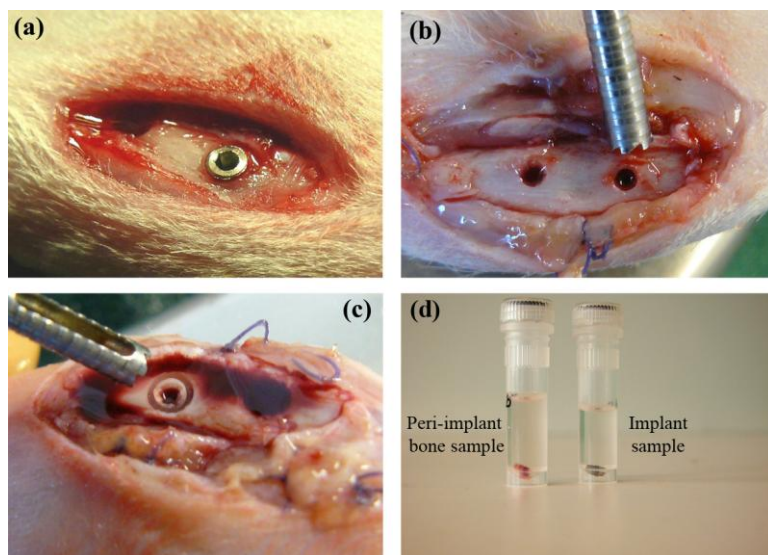


Figure 8.5. A photograph of the retrieval procedure for gene expression analysis of implant-adhesion cells. (a) Unscrewing the implant. (b, c) Retrieving the peri-implant bone using a trephine. (d) RNase-free tubes are used to collect the bone samples and the unscrewed implants for qPCR analysis.

Implants are unscrewed with adherent biological material using a screwdriver. A trephine is used to retrieve the peri-implant bone (Figure 8-5). RNase-free tubes are used to collect the qPCR samples, which are stored until the analysis is performed. (Please see Chapter XV for more detailed information).

8.5.2. Extra-Oral Implantation Model

Pre-clinical models of long bones, which are of endochondral origin, are often used in histologic evaluations of dental implants that are going to be clinically placed in jawbones of membranous origin. In addition, the jawbone remodeling pattern and blood supply are different from those of endochondral long bones. For this reason, the parameters used to evaluate osseointegration in long bones may not be optimal when it comes to evaluating craniofacial osseointegration. Some researchers therefore developed small laboratory experimental models for the observation of tissue responses to biomaterial implantation in jaws. They confirmed the usefulness of extra-oral and intra-oral approaches in the small laboratory animal models as a pre-clinical model for dental implant and guided tissue regeneration research. [46-48].

The extra-oral approach provides isolation from the oral environment and is thus able to prevent negative effects such as contamination and infection by saliva and other microorganisms. One of the common extra-oral models for guided tissue regeneration is the transosseous skull-bone defect model in both rat and rabbit models. However, skull bone consists mainly of cortical bone. The opportunity to test different barrier membranes and/or bone substitutes and study their behavior in trabecular bone and bone marrow during healing is therefore limited when using this model. As a result, it may be preferable to use the jawbone area. However, the creation of discontinuity defects in the mandibles of small laboratory animals is difficult because of limited surgical access. As a result, through-and-through defects of the mandible may be possible only in the mandibular anterior and ramus area of small animals.

In 1988, Dahlin and his co-worker were the first to apply the Guided Bone Regeneration (GBR) principle to the treatment of standardized, 5mm in diameter, transmandibular defects in rats. [48] In a subsequent experimental study, a similar model of mandibular critical size defects in rats was used in order to evaluate GBR treatment efficacy following the application of resorbable membranes.

The surgery can be performed on both sides of the mandible. The incisions are made on the outer skin and the dissection is continued through the subcutaneous tissue and muscle, 5mm coronal to its inferior border. The masseter muscle is exposed and a muscle-periosteal flap is elevated from the mandibular ramus with a periosteal elevator, leaving the bone exposed. An osteotomy is performed using a round bur. The custom-designed titanium implants with a diameter of 2.8mm and a length of 4mm were placed bilaterally. On one side of the jaw, chosen at random, the (test) implant was placed, while, on the other side of the jaw, a similar (control) implant was placed (Figure 8-6). Because of the small size of the rat mandible, one disadvantage of this model is that surgery is only possible in the mandibular ramus area.



Figure 8.6. A photograph of a titanium bone screw (1.7mm in diameter and 3mm in length) placed in the lateral aspect of the rat mandible.

In the rabbit model, the extra-oral approach in mandibular bone is rarely used in implants and bone augmentation research and it has usually been combined with the intra-oral approach as a prerequisite for extracting the anterior teeth. [49].

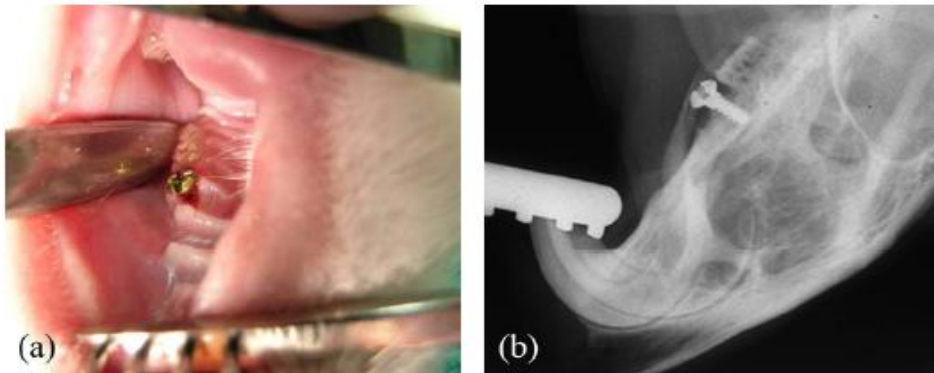
Surgery is performed by extracting the bilateral mandibular incisors during the initial surgical intervention, because the rabbit mandibular body is occupied by teeth and there is no room for defects. Sockets from which the teeth have been extracted are allowed to heal for 2 months before the second surgical intervention. At the second surgical intervention, an extra-oral incision can be used to expose the anterior mandible and create two similar-sized bone defects bilaterally in the inferior border anterior to the mental foramen with a high-speed bur under saline irrigation. The defect can be filled with bone grafts and fixed in the site by one implant on each side. One hole is drilled in the center and penetrates through the bone graft into the host bone. The holes are made with drills that gradually increase in diameter. The bone preparation is performed using a low rotational drill speed (450 rpm) with continuous internal cooling. The bone cavities are washed with saline during and after the drilling.

The advantages of the extra-oral approach are good access to the implantation site, less trauma, the avoidance of tooth fracture during the extraction process and the minimization of the likelihood of oral contamination and thereby infection.

8.5.3. Intra-Oral Implantations Model of Dental Implants under Loading Conditions

This subtitle takes a leap of faith by suggesting that a titanium mini-screw placed in the maxilla of a rat is the equivalent of a human dental implant, which it is not. While the titanium of such an appliance is often of the type designed for placement in bone, and the size

is roughly to scale for the smaller animal, several key differences between the rat model and the human are significant and must be considered when interpreting any results obtained from this model. The main difficulty of oral implantation in small animals is that of access. In the rat model, the mandible could be depressed and the lips retracted to present adequate access for all operations, and this animal was considered to be the most satisfactory for ease of handling, control of anesthesia, and natural resistance to infection. The experience of the authors with the placement of mini-screws to model dental implants has produced the following protocols: Before implant placement surgery, all rats are administered an intramuscular anesthetic. For each rat, the first molar of the left maxilla is extracted. Extraction sites are allowed to heal for one month before implant placement. [12] After the healing period, a full mucoperiosteal incision is made on the left side, mesial to the second molar on the top of the ridge to expose the bone. Using a low-speed hand piece, a hole is drilled under irrigation (provided from a transfer pipette) directly on top of the ridge parallel to the second molar. After confirming the sinus floor is not penetrated, a titanium mini-screw (Stryker Leibinger, Kalamazoo, MI) with a length of 3 mm and a diameter of 1.2 mm is placed perpendicular to the maxillary occlusal plane (Figure 8-7). The implant head is adjusted horizontal to the occlusal plane with a screw driver. For the loaded groups, the implant head is further adjusted to contact the first molar of the mandible using articulation paper. The left first molar of the mandible is extracted for the unloaded group. No attempt is made to cover the mini-screws after implantation.



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Figure 8.7. (a) Rat model showing titanium mini-screw implant after placement in the area of a healed first maxillary molar extraction. (b) Radiograph showing micro-screw implant placement and orientation. Note: screw head has been adjusted into occlusion.

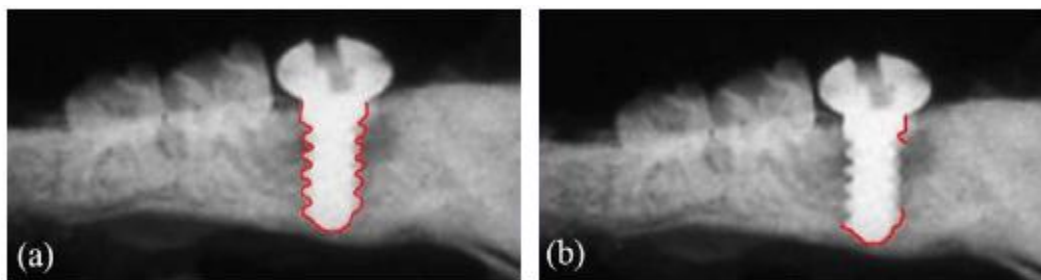
8.6. Quantitative Analysis of *Bone-Implant Interface*

8.6.1. Bone/Implant Contact Measurements

Bone implant contact measurements can be taken using standardized soft radiographs (12 sec with 2.5 mA and Kvp of 50) (either film or digital) of the maxilla of anesthetized animals

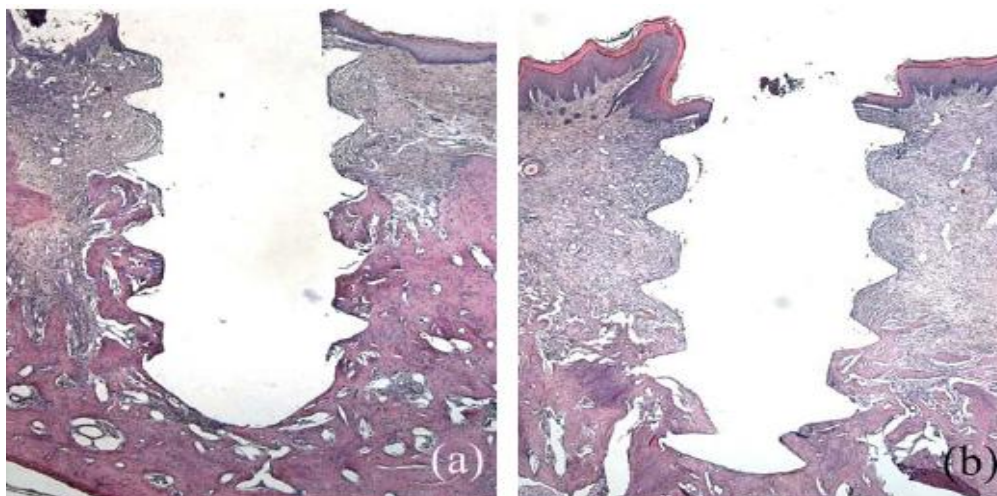
with their heads mounted in a fixed positioning device and using a Faxitron Series 43807N x-ray system (Hewlett-Packard, Palo Alto, CA) or similar system for exposure at the day of implant placement, and at time intervals of up to 12 weeks after placement (Figure 8-8).

The UTHSCA Image Tool software (San Antonio, TX) or similar software is used to estimate bone contact ratio on the scanned x-rays from each time point. The bone/implant contact ratio is determined by measuring the implant segments in contact with x-ray opaque areas of presumed bone and dividing the sum of these segment lengths by the total implant thread length. These measurements are taken three times for each implant at each time point. Measurements are standardized by adjusting the value of the initial measurement for each implant at the time of implant placement as equaling a bone/implant contact ratio of 1.0. All measurements for each implant across time are related to their initial adjusted value.



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Figure 8.8. Estimated bone/implant contact ratio determination from x-rays. (a) Total implant length determination. (b) Bone/implant contact length determination.



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Figure 8.9. A. Hematoxylin and eosin (HandE) stained section of tissue surrounding a loaded rat microcrew implant after 21 days. This example shows bone associated with approximately 2/3 of the implant surface. B. HandE stained section of tissue surrounding an unloaded rat microcrew implant after 21 days. This example shows bone associated with the basal 1/3 of the implant surface.

This is characterized as the estimated contact ratio because of the limited resolution of the x-rays. The estimated contact ratio determination from the soft x-rays, may also be compared to the contact ratios determined using the tissue sections (from time points where these are available) for each implant after processing (Figure 8-9). A correlation of the results obtained by both techniques can be used to validate the estimated contact ratio data. [12].

8.6.2. Micro-CT Assessment

With the availability of the Micro-CT, bone/implant contact ratios and many other parameters related to osseointegration can be measured in the rat model. Experimental and control rats are euthanized under deep anesthesia using an intramuscular injection of ketamine (100 mg/mL), xylazine (20 mg/mL), and acepromazine (10 mg/mL). The animals are perfusion fixed with 60 mL of 10% neutral buffered formalin (Sigma, St. Louis, MO) and the maxilla containing each implant is dissected for micro CT analysis (SkyScan 1174, SkyScan, Kontich, Belgium) using an Isomet low speed diamond saw (Buehler, Lake Bluff, IL). Micro CT can be used in order to avoid artifacts due to specimen orientation by creating 3D reconstructions for analysis. Our previous experience with this instrument has yielded excellent results for micro CT analysis of bone while samples are submerged in formalin. Appropriate calibration of the instrument must be done before each measurement session with bone phantoms of known density submerged in formalin. Some correction must be included in the analysis to account for reflections of the x-ray beam off of the implant surface. A recent study by De Smet used specially designed hollow implants to reduce this effect. [16].

The parameters measured are based on the nomenclature of Parfitt *et al.* [50] For measurement and comparisons between treated and control groups, measurement parameters may include: *bone mineral density* measured in Hounsfield units; *bone volume* in mm³; *bone surface density* as a ratio of surface area to total volume; *trabecular thickness* in mm; *trabecular number* defined as the number of traversals across a trabeculae or solid structure per unit length on a linear path through a trabecular bone region; *Euler number* to determine the interconnectiveness of the 3D trabecular structure; *degree of anisotropy* which represents asymmetry or the absence of preferential alignment of structures along a particular directional axis; *fractal dimension* as an indicator of surface complexity or space filling capacity (there are no units);[50] and *porosity* which is a measure of fully enclosed spaces as a percent of the total area. Following micro CT analysis of bone morphometric parameters, all maxillary specimens are usually transferred from formalin to a decalcification solution consisting of 10% EDTA in H₂O at pH 4.5-5 for 1 month. Decalcified specimens may then be processed for routine histology.

8.6.3. Biomechanical Tests

Bone response to implanted biomaterials is traditionally evaluated and quantified by histologic and histomorphometric techniques. However, the histologic procedures do not provide information about bone-implant interfacial strength.

Various biomechanical tests have been introduced to evaluate the bone-implant interfacial strength and they include pull-out, push-out, tensile and removal torque tests.

However, the lack of consistency between animal models and geometric implant designs seriously questions the consistency of real-time biological data. Further, bone exhibits considerable variations in its structure, as a result of species, age, environment and function, which leads to significant differences in modulus, strength and toughness in both cortical and cancellous bone.

The shape or the geometric implant design of the implant is therefore always an additional issue in the selection of a biomechanical test. For this reason, push-out testing requires the use of cylindrical implants. In contrast, when removal torque is used to test the screw-shaped implant design, the fracture interface should also be analyzed after torque testing, to determine whether the torque failure is indeed caused by the failure of the bone-implant interface.

8.6.3.1. Pull- and Push-Out Tests

Push-out testing has been shown in the literature to be a commonly reported test for evaluating implant bonding to bone. The simplicity is the reason why these tests are preferred when it comes to assessing the bone-implant interfacial strength. However, several authors dispute the suitability of these tests for assessing bone-implant interfacial strength. [51-53] They believe that the results from this method are either incorrect, as a result of misalignment between the direction of loading and the interface, or misleading, due to measurement of the forces necessary to overcome both bone bonding and mechanical interlock, especially when comparing materials with varying surface parameters such as roughness, or porosities.

The same authors promote tensile type tests, in which the main axis of loading is perpendicular to the implant/tissue interface.

Brånemark noted that pull-out and push-out tests measure peri-implant bone quality, since, by definition, the bone surrounding the implant fractures during the experiment. [54].



Figure 8.10. A photograph of the removal torque tester holding the sample. A special hexagonal screwdriver, connected to the torque test machine, was fitted into the implant internal hexagon.

Although the push-out test measures the mechanical properties of bone around the implant and the strength of the coating-implant interface rather than measuring the shear strength of the bone-implant interface, from a clinical perspective, push-out data still provide valid information about how strongly an implant is anchored in the bone.

8.6.3.2. Removal Torque Test

In 1987, Johansson and Albrektsson introduced the removal torque force as a method for biomechanically measuring anchorage or osseointegration [55] in which the larger forces required to remove implants may be interpreted as an increase in the strength of osseointegration (Figure 8-10). They suggested that increases in the ‘removal torque value’ are correlated to the increase in the bone-implant contact area along the interface.

8.7. Assessment of Loading in the Rat Intraoral Implant Model

Osseointegration may be defined as a direct structural and functional connection between ordered living bone and the surface of a load-carrying implant. [58] It is reported that large strains can damage bone by causing micro-motion early after implantation which interferes with local bone healing and predisposes the implant to a fibrous tissue interface instead of osseointegration. [59-61] Other authors suggest that implant loading, when appropriately controlled can stimulate bone remodeling around the implants that is beneficial to maintain implant stability during the early critical healing period. [62-64].

8.7.1. Measurement of Rat Bite Force

A technique described by Byron et al. to measure bite force can also be used as a method to load rat intraoral implants *in vivo*. [65] This technique (as adapted from Byron) uses a Grass SD9 stimulator (Astro-Med Inc., West Warwick, RI) with platinum electrodes to deliver field stimulations to the temporalis muscle. A 1 ms square wave pulse of 10 s duration over a 0–18 V range and frequency variation of 2–20 Hz was used in his published study, but can be adjusted as needed. [65] A 10 s recovery period was also used between each successive stimulation voltage to allow time for muscle recovery.

Integral force, as determined by the area under the bite force curve, was measured in Newtons using a subminiature load cell (Sensotec Honeywell, cat. #AL322, Columbus, OH) placed between upper and lower incisors. This measurement can be used to standardize the experimental load for comparisons between groups.

8.7.2. Finite Element Analysis

Recent studies suggest that a better understanding of the *in vivo* bone response to loading can be achieved by mapping actual stress-strain distributions at the bone/implant interface. Mapping of this distribution in conjunction with the putative triggering effects on cells and

biological activities in normal and medically compromised animal model could serve as an aid for determining patient selection criteria and implant design.

The biomechanics of the bone surrounding dental implants has been analyzed by both 2-dimensional (2D) and 3-dimensional (3D) finite element analysis (FEA) methods. [66-72] The use of FEA in implant biomechanics permits the simulation of the complexity of mechanical forces encountered in clinical situations. [71].

The limitations of this method are primarily that it is sensitive to the assumptions made regarding model parameters. [68] In most FEA models, the implants are assumed to be 100% osseointegrated. [66, 70-72] Some histomorphometric and FEA studies, however, indicate that there is never a 100% bone-implant interface. [70, 72].

In studies from the author's laboratory, 2D-FEA modeling on histological specimens from loaded implants was used to provide information about the correlation between mechanical stress and tissue responses. [12] The type of cellular detail observable in tissue sections for routine histology and immunohistochemistry makes this method of 2D-FEA overlay a valuable correlate to 3D-FEA studies which are not as readily comparable to the histological changes at the cellular level.

Rats with mini-screw implants for histological or 2D-FEA are perfused transcatheterially with 100 mL of 10% formalin under deep anesthesia. The maxilla with the implant is dissected and sectioned using a low-speed saw (Isomet Buehler Ltd., Lake Buff, IL) under constant water irrigation supplied by dripping the solution from a transfer pipette. The samples are demineralized for six weeks in 0.1 M EDTA and 0.1 M NaOH and the implants from the samples are removed after decalcification for paraffin embedding, or left in the maxilla for plastic embedding. The bone blocks are then processed and sectioned by routine histological methods.

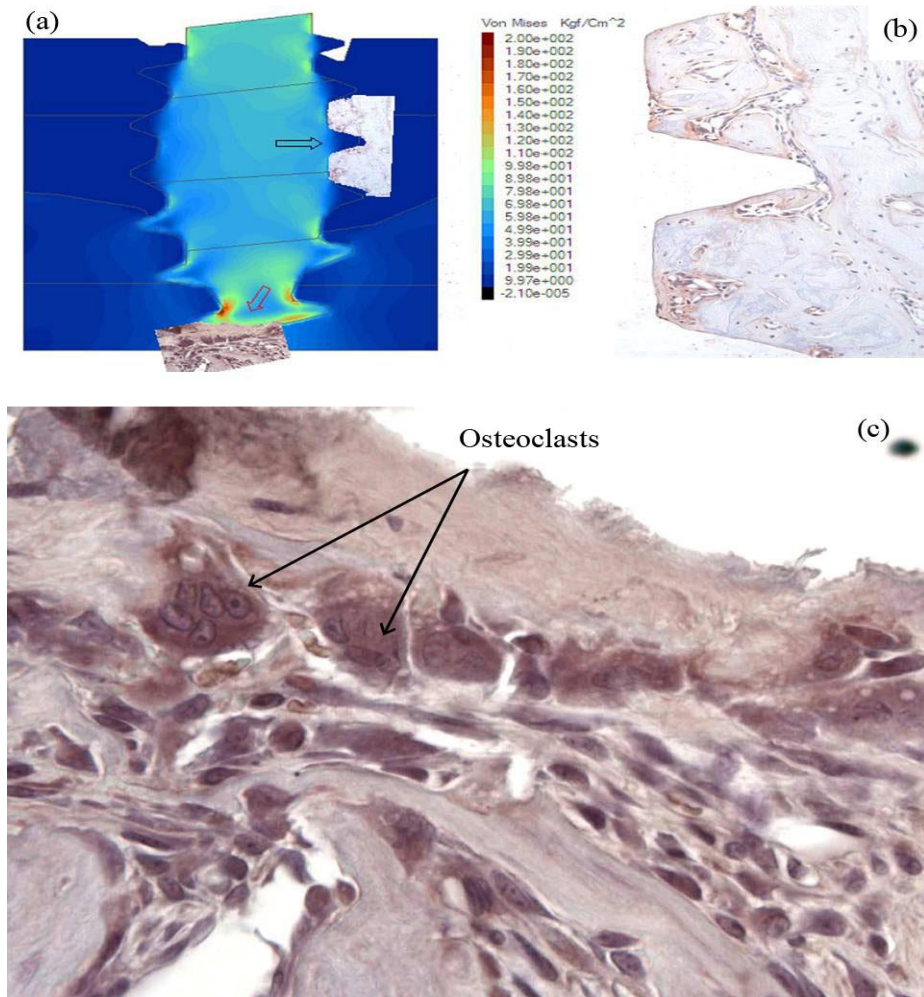
Stained sections from loaded implant groups are scanned and imported into the WinTopo program (SoftSoft.net, Biggleswade, Bedfordshire, UK), which vectorizes and saves images as dxf files. In this format, FEA can be performed on the images using the J.L Analyzer 9.0 FEA program (AutoFEA Engineering Software Technology Inc., Norwalk, CA).

With this software, 2D-FEA models are constructed containing between 2000 and 4000 nodes or connections between the elements of the model. Models are constrained on the proximal, distal and basal surfaces and a 1 Newton load (or other tested load) is applied in the -y direction on the occlusal surface. [73] Young's moduli adopted from the literature, [74-76] give values for soft tissue of 0.6MPa, bone (mean) of 2000 MPa, and titanium of 110,000 MPa, as shown in Table 8-2. A Poisson's ratio of 0.3 was used for soft tissue, bone, and titanium. All materials are assigned isotropic, linear elastic properties.

Table 8.2. Mechanical parameters of soft tissue, bone, and titanium used in the author's study

Material	Young's modulus (MPa)	Poisson's ratio (μ)
Soft Tissue	0.06	0.3
Bone (mean)	2000	0.3
Titanium	110000	0.3

The values for soft tissue and bone were taken from Choi and Zheng [74] and Kavarizadeh *et al.* [77].



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Figure 8.11. (a) Two overlays of MMP-13 stained sections from 21 day loaded implants above the von Mises shear stress map derived from a serial histological section of the same implant. The first overlay (black arrow) shows osseointegration in the central region of the implant where MMP-13 localization is minimal and shear stress is near zero (~ 0.97 MPa). A second overlay (red arrow) from the base of the implant shows an area of high stress (10 MPa to 120 MPa). B. Enlargement of the low stress area adjacent to the implant showing well formed bone and minimal MMP-13 staining. C. Enlargement of the high stress area at the base of the implant showing multiple osteoclasts and MMP-13 staining (brown color).

In all FEA analysis slight changes in constraint or other parameters will have significant effects on the final model. The resulting stress maps created for each section are overlaid with the immunohistochemistry image of the section used for the FEA construction using the Adobe Illustrator software program (Adobe Systems, San Jose, CA).

This technique permits the characterization of the load environment as it relates to the tissue characteristics and the localization of molecules by immunohistochemistry and in-situ hybridization (Figure 8-11).

Statistical Analyses

Estimated contact ratios and other bone parameters can be compared using ANOVA or Student's t comparisons of the means between groups. The correlation coefficient also may be used to compare the bone/implant contact ratio determinations at obtained from the x-rays to values determined from the histological sections.

Conclusion

A number of models and methods commonly used for dental implants and osseointegration researches in small laboratory animals have been described. The advantages and limitations of these models have been discussed.

The use of titanium mini-screws in the rat maxilla is a new technique for modeling dental implant osseointegration. The limitations of this technique rest primarily in the differences between rat bone and human bone and in the differences between titanium mini-screws and human dental implants and their placement. With the increasing popularity of titanium mini-screws for temporary orthodontic anchorage, the development of small titanium implants with thread designs and surfaces that more closely resemble human dental implants are positioned to significantly improve this model.

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Chapter IX

Animal Models for Alveolar Bone Augmentation

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9.1. Introduction

The literature consistently supports the concept that periodontal disease can be arrested and that even teeth with extensively reduced periodontal support, if adequately treated, may be maintained. [1-3] However, there are a number of situations where a tooth is already lost or compromised beyond rehabilitation, leaving few remaining treatment options. For these situations, implant dentistry has become an attractive, if not preferable, alternative to replace compromised or missing teeth. Implant dentistry has shown high survival and success rates in both edentulous and partially edentulous patients [4] and provides significant improvement in quality of life. [5-7] The first prerequisite for predictable long-term success of implant dentistry is the presence of an adequate geometry and volume of alveolar bone to provide functional anchorage for implant fixtures. However, in many situations this prerequisite is not met, residual alveolar bone is often insufficient to allow (optimal) therapy.

In the recent past, placement of implant fixtures was primarily guided by the morphology of the edentulated alveolar ridge. Resective procedures were used to fit implant fixtures to the residual alveolar bone, and cantilevers were often used where implant fixtures could not be placed. However, patients require dentists to address not only functional but also aesthetic demands. Advances in alveolar bone augmentation technologies and procedures today allow treatments directed to avoid fenestrations and dehiscences and to provide adequate bone volumes and in extension soft tissue profiles that in turn may produce a more natural appearance. These advances also require development of bone inductive technologies

including biomaterials, devices, and bone growth factors to accommodate an increasing demand for safe and effective restoration of the alveolar bone but also soft tissue augmentation. In addition, longevity is increasing the population requesting implant dentistry.

Contemporary bone augmentation procedures are based on general principles of tissue repair. However, this knowledge does not necessarily guarantee the safety and efficacy of a candidate biomaterial or biologic construct for reconstruction of the deficient alveolar ridge. Although use of *in vitro* models may clarify mechanistic biological events, stand-alone, they are insufficient and inadequate to evaluate new treatments. *In vivo* preclinical models provide an increased comprehension of the biological processes that occur in response to the delivery of a biomaterial or biologic construct. [8-9] Moreover, most preclinical studies include invasive procedures such as collection of block biopsies for histologic and micro-CT analysis, which cannot be performed in humans. Taken together, the use of preclinical (animal) models thus represents a necessary step in the development and evaluation of a candidate therapy for alveolar augmentation prior to human application.

9.2. The Triple R Concept

Russell and Burch introduced the Triple R (*Reduce, Refine and Replace*) Concept in their text *The Principles of Humane Experimental Technique*. [10] The Triple R Concept aims to 1. *Reduce* the number of animals used in research; 2. *Refine* procedures to minimize pain and discomfort to animals used in research; and 3. *Replace* animal studies with alternative protocols for *in vivo* evaluations. Initially, the Triple R Concept was not widely implemented; however, laws and protocols have been created establishing a broad adoption of this concept for studies using animals for research.

Researchers can reduce the number of animals by avoiding replication of *in vivo* studies, improving data acquisition, decreasing sources of experimental variability, and obtaining the largest possible number of relevant observations from a small number of animals. In addition, and perhaps the most important factor to reduce the number of animals is the use of appropriate *discriminating models*, avoiding ineffective models, which are not capable to define the biological potential of a new therapy. The *Refine* term refers to use methods that alleviate or minimize potential pain and distress and enhance animal well-being. These methods include a period of acclimation; use of cages, food and light cycles that minimize stress; use of best available surgical techniques; use of anesthetics, analgesics, and antibiotics whenever is necessary; and frequent observation of the animals by trained veterinary staff. In this regard, the health and well-being of the animal precede the aims and results of the studies. The *Replace* term refers to methods that permit a given purpose to be achieved without conducting scientific procedures using animals. For instance, *in vitro* studies, purpose-designed computer simulations or accumulated data from previous experiments (systematic reviews). Additionally, phylogenetically less developed animals should be used whenever possible. The education and training of researches and veterinary staff, the full implementation of existing laws, a continuous dialogue among all parties involved (scientists and laymen alike) bring scientific, economic and humanitarian credence to and address the fundamental principle of Russell and Burch's concept: scientific excellence and the greatest humanity in the use of laboratory animals.

9.3. Animal Platforms, Defect Models and Outcomes

In bone research, the animal species should be regarded a platform in which different defects can be created to study biological principles or serve as clinical surrogates. Nonhuman primates, pigs, dogs and rats have morphological, physiological and behavioral differences inherent to each species that make them more suitable for a specific study. For instance, pigs are not suitable for studies in which constant manipulation of the oral cavity is necessary since multiple anesthesia and sedation procedures may deleteriously affect animal health. Different experimental interventions and situations may be created in a given animal platform to study a specific biological mechanism and/or clinical situations. For instance, inlay or onlay defects may be created in the dog mandible to serve as surrogates for defects observed in patients. These concepts can be further extended by modifying the animal platforms or the defect models to address biological or clinical peculiarities. Genetically-modified or experimentally-induced diseased rats may be used to study the effect of specific conditions (diabetes, osteoporosis, etc) on bone formation using the critical-size calvarial defect model. Similarly, mandibular defects may be created in the type I/II or III/IV bone depending on location (anterior vs. posterior) and surgical manipulation (removal of cortical bone).

9.4. Criteria for Suitable Animal Platforms and Defect Models

Certain criteria appear of particular importance and should be carefully considered before selecting an animal platform/animal model to evaluate alveolar augmentation. A preferred platform/model should be cost effective, easy to handle and constant allows post-operative evaluation and monitoring, and provide meaningful information that ideally can be used for a wide range of clinical applications/indications. Furthermore, a preferred platform/model should also be stable, offer minimal complications during the surgical procedures; and provide anatomic/morphological features/characteristics and dimensions that enable the application of restorable devices, biomaterials and biologic constructs of similar if not identical dimension and design projected in the designated clinical population.

An important aspect that should be considered is the biological relevance of the platform/model. Knowledge of peculiarities of alveolar bone appears critical for the choice of an ideal platform/model. Bone is a dynamic, highly vascularized tissue with innate regenerative potential. The skeletal system is permanently remodeling to meet functional and metabolic demands. At any given time, 0.6% cortical and 1.2% cancellous bone undergo surface resorption, whereas 3% cortical and 6% cancellous bone undergo surface formation. [11] Although fundamentally comparable to other skeletal domains, alveolar bone presents unique and distinguishing features. Embryologically, alveolar bone is derived from cells of the neuroectoderm germ layer similar to other craniofacial bones but distinct from the axial and the rest of the appendicular skeleton originating from the mesoderm. [12] In addition, while the alveolar bone is formed through intramembranous bone formation, the remaining skeleton is formed via an initial deposition of a cartilaginous template subsequently replaced

by bone; i.e., endochondreal bone formation. Another distinguishing feature is the turnover rate of the alveolar bone, which appears more rapid than in any other part of the skeleton. Continual and rapid remodeling is associated with tooth eruption, functional demands, and in response to the physiological movement of teeth that occurs with the development of the jaw. The periosteum and endosteum rearrange to accommodate the external and internal architecture of the alveolar bone to tooth migration. These mechanisms result in continuous plasticity of the alveolar bone around erupted and functioning teeth, so the tooth socket can be considered a structural element that is never stable. [13] Moreover, tooth loss results in bone atrophy and conspicuous resorption of the alveolar ridge in a chronic gradual order, [14] making placement of dental implant fixtures a challenge. Taken together, it appears that unique features of alveolar bone may with difficulty, if at all, be reproduced at other skeletal locations. Consequently, studies directed to evaluate candidate therapies for alveolar augmentation should be performed using alveolar bone models and not skeletal surrogates.

9.5. Small and Large Animal Platforms and Defect Models

A candidate therapy for alveolar augmentation and also bone regeneration in general should first be evaluated for biologic safety and potential using screening platforms/models prior evaluation of efficacy and clinical potential, and in turn clinical adoption. Such platforms/models include ectopic and orthotopic rodent models. [15-18] Rodents (mice, rats and rabbits) are the most commonly used animals in biomedical research, mainly because they are cost-effective, easy to handle, and allow for standardization of experimental conditions in genetically similar individuals. The most common and well-documented defect model for evaluation of devices, biomaterials, and biologic constructs to support bone formation is the rat critical-size through-and-through calvarial osteotomy defect. [19,20] Critical-size calvarial defects have also been described for different animal platforms such as rabbits, sheep, minipigs and nonhuman primates, however, the use has been somewhat limited. Therapies that show biological potential in such qualified screening platforms/models should be evaluated for efficacy and clinical potential in discriminating large animal models, predominantly using canine or nonhuman primate platforms. Dogs have been shown a highly appreciated platform for bone regeneration research. In view of their extremely cooperative temperament, ease-of-handling, straightforward housing, and relatively low cost compared with nonhuman primates, they have become the preferred choice for study alveolar bone augmentation. Moreover, dogs present features close to humans relative to bone weight and density, as well as bone constituents including organic, inorganic and water fraction. [21] However, they also present some differences including higher rates of trabecular and cortical bone turnover. Nonhuman primates, on the other hand, are closer phylogenetically to humans. They present a wide range of sizes, from small marmosets to sizes similar to the human, including chimpanzees and gorillas. Their tooth anatomy is close to that of humans; however, still presents striking dissimilarities. Limitations for the use of monkeys as a platform for research include high cost, probability of disease transmission, and importantly low cooperation. Although some studies using large animals have failed to accurately predict the outcomes in human trials, it remains a primordial step before using any therapy in humans.

9.6. Alveolar Defect Models

Essentially, alveolar defect models can be divided into two categories: inlay and onlay defects. Typical examples of inlay defects are tooth extraction sites, periapical defects and alveolar ridge saddle-type defects, all defects within the alveolar envelope. Inlay defects present multi-directional vascular and cellular tissue sources in support of bone regeneration. In contrast, vascular and cellular sources for bone regeneration within onlay defects origin from the limited alveolar base of the defect.

Although inlay defects represent common clinical situations, their capacity as experimental models to discriminate clinically meaningful therapies for alveolar augmentation is unconvincing. For example, Araújo *et al* used extraction sites in Beagle dogs to evaluate the potential of a cadaver-sourced bovine bone biomaterial (Bio-Oss®) to enhance bone healing in peri-implant gap defects, inlay defects that occur adjoining an implant fixture placed into a wider extraction socket. [22] Sites receiving the biomaterial and the sham-surgery control without the biomaterial healed almost completely within the same time frame. A similar innate healing potential was observed for large surgically created implant dehiscence defects in the *Cynomolgus* monkey when evaluating recombinant human bone morphogenetic protein-2 (rhBMP-2) in an absorbable collagen sponge (ACS) carrier versus sham-surgery. [23] Bergenholtz *et al.*, also using *Cynomolgus* monkeys, evaluated the potential of rhBMP-2/ACS to accelerate bone healing in large access defects for periapical endodontic surgery. [24] The defects healed almost to completion within a 4-week healing interval. Large alveolar ridge saddle type defects also lack a convincing discriminatory potential. Evaluating the bone inductive potential of rhBMP-2/ACS, Jovanovic *et al.* observed a 60% bone fill in the sham-surgery control versus 100% for the rhBMP-2/ACS; this leaves limited room to demonstrate osteoconductive and/or osteoinductive potential of a candidate biomaterial or biologic construct. [25] Onlay models, on the other hand, appear useful tools for evaluate candidate osteoconductive and/or osteoinductive therapies, since they have a limited innate osteogenic potential. A series of reports using the critical-size supraalveolar periodontal defect model [26-28] and the critical-size supraalveolar peri-implant defect model [29,30] demonstrated the limited native regenerative potential of these models, subscribing to the concept that a discriminating model must present minimal innate regeneration to convincingly allow evaluation of the biological potential of a candidate therapy for alveolar augmentation.

Summarizing the contrasting observations between inlay and onlay defects, it appears that the defect type by far overshadows the choice of animal platforms within the framework of large animal species to evaluate alveolar augmentation. Thus, unless situations where some systemic condition (such as diabetes or osteoporosis) is the main focus of the study, the animal can be considered a platform where the new protocol, biomaterial or biologic construct is tested, while the defect type represents the actual model to evaluate the osteoinductive/osteoinductive potential of the candidate technology. Important and in addition to all aspects already discussed is the reliability of the model. A high standardization of the procedures, with clear landmarks and meaningful recordings are necessary. Finally, well-documented evidence evaluating the potential of different therapies using the platform/ model should be available.

9.7. The Critical-Size Defect Concept

Historically, the critical-size defect has been described as the smallest osseous defect that will not spontaneously heal over the lifetime of an experimental animal without adjunctive measures. [20] However, most studies are of limited duration and do not extend over the lifetime of the experimental animals, thus for all practical reasons the critical-size defect is commonly referred to, and perhaps even misunderstood as, a defect that will not heal over the duration of the study without adjunctive measures (sham-control). Researchers selecting/developing such models thus must be cognizant to carefully match defect dimensions to observation interval to benefit from a valid experimental design. This alternative paradigm in turn may demand a somewhat altered interpretation of investigated biologic processes. There probably are innate differences in regenerative potential among non- and slowly healing bone defects versus normally healing defects that do not have sufficient time to heal. [31] Furthermore, not only the size of the defect but also by the manner in which it was created and surgically managed appears important when considering the innate biological potential. For instance, age/systemic conditions of the animals; nutrition; local bone structure (chronic vs. acute defects) and vascularization are some of factors that may play important roles in wound healing/bone regeneration.

Defect models for alveolar bone augmentation in various animal platforms are mostly not well-described, defined or standardized making comparison among studies difficult if not treacherous. In view of the substantial research directed to the development and evaluation of novel devices, biomaterials, and biologic constructs for bone repair and regeneration, the need for standardized defect models appears essential to produce reliable data that can be evaluated and compared retro- and prospectively. In perspective, researchers should focus their creativity on designing new concepts and therapies instead of changing surgical protocols and defect configurations which renders most studies incomparable, therefore of uncertain value. The critical advantage of a critical-size defect model depends on the accuracy and reproducibility of the model, which in turn rests on a standardized defect configuration and limited biologic variability. This reproducibility is only achieved if the defect model is carefully considered, described and tested. Experimental design, surgical protocols, tissue preparation, histological and radiographic analysis must be reported in detail.

9.8. The Critical-Size Supraalveolar Peri-Implant Defect Model

The critical-size, supraalveolar periodontal defect model using an adult male Beagle dog platform has been extensively described and characterized by Wikesjö and co-authors. [26-28, 32-36] In subsequent studies skeletally larger, adult male Hound Labrador mongrel dogs were used mainly due to their size, but also due to their favorably cooperative behavior, which allows daily/regular/frequent post-operative intraoral care and review. The general principles from the supraalveolar periodontal model were carried forward to study alveolar augmentation and dental implant osseointegration introducing the critical-size, supraalveolar, peri-implant defect model. [37,38] This model has received extensive recognition and use to

study a range of growth factors, carrier matrices, devices and biomaterials including demineralized bone matrix — DBM, [29] recombinant human bone morphogenetic protein-2 — rhBMP-2, [39-49] rhBMP-7, [50,51] and recombinant human growth/differentiation factor-5 — rhGDF-5. [52] The critical-size, supraalveolar, periodontal defect model and the critical-size, supraalveolar, peri-implant defect model have thus become a “litmus test” for candidate therapies for periodontal wound healing/regeneration and alveolar augmentation/osseointegration and have been used exhaustively, generating more than 50 publications concerning a wide range of candidate therapies.

9.8.1. Experimental Surgery

Food is withheld the night preceding surgery. The animals are pre-anesthetized with atropine (0.02-0.04 mg/kg IM), buprenorphine HCl (0.01-0.03 mg/kg IM), and acepromazine (0.2-0.3 mg/kg IM). After tranquilization, a 20/23-gauge intravenous catheter is placed in the foreleg for induction with propofol (5-7 mg/kg IV). Animals are intubated with an appropriately sized (7-9 mm) endotracheal tube and then moved to the surgical theater to be maintained on gas anesthesia (1.5-2% isoflurane/O₂ to effect). The animals receive a slow constant rate infusion of lactated Ringer’s solution (10-20 ml/kg/hr IV) to maintain hydration during surgery. Depth of anesthesia is monitored evaluating response to toe pinch, corneal reflex, and by monitoring the depth of respiration, respiratory rate and heart rate.

Following routine general and conventional local infiltration anesthesia (lidocaine HCl 2%, epinephrine 1:100,000) buccal and lingual mucoperiosteal flaps are reflected and alveolar bone removed around the mandibular premolar teeth to a level approximately 6 mm from the cemento-enamel junction using water-cooled rotating burs. The premolar teeth are then extracted and the 1st molar amputated at the level of the reduced alveolar crest. The alveolar process is then carefully planed using large water-cooled rotating burs to create a plane facilitating precision dental implant placement. Next, following osteotomy preparation, the ø4.0-4.3/10-mm dental implants are placed into the extraction site of the distal root of the 3rd premolar and into the mesial and distal root of the 4th premolar in each jaw quadrant. When in unique cases implant placement into extraction sites is not possible, implants are placed into primary osteotomies prepared into the reduced alveolar process positioned to match designated extraction sites. Five mm of the ø4.0-4.3/10-mm implant is placed within the surgically reduced alveolar crest, leaving the rest of the implant above the crestal plane, thus creating the 5-mm, critical-size, supraalveolar peri-implant defect. The model presents clear landmarks for effective surgical execution. Experimental and control treatments are randomized to/alternated between left and right jaw quadrants in subsequent animals with only one treatment/jaw quadrant. Next, the periosteum of the mucogingival flaps are fenestrated at the base of the flaps to allow coronally advanced tension-free flap apposition and wound closure submerging the implants and any implanted device, biomaterial or biologic construct. The flaps are advanced 3-4 mm coronal to the implants and the flap margins adapted and sutured preferably using expanded polytetrafluoroethylene/ePTFE sutures (GORE-TEXTTM Suture CV5, W.L. Gore and Associates Inc. Flagstaff, AZ, USA) and mattress suturing techniques (Figure 9-1).

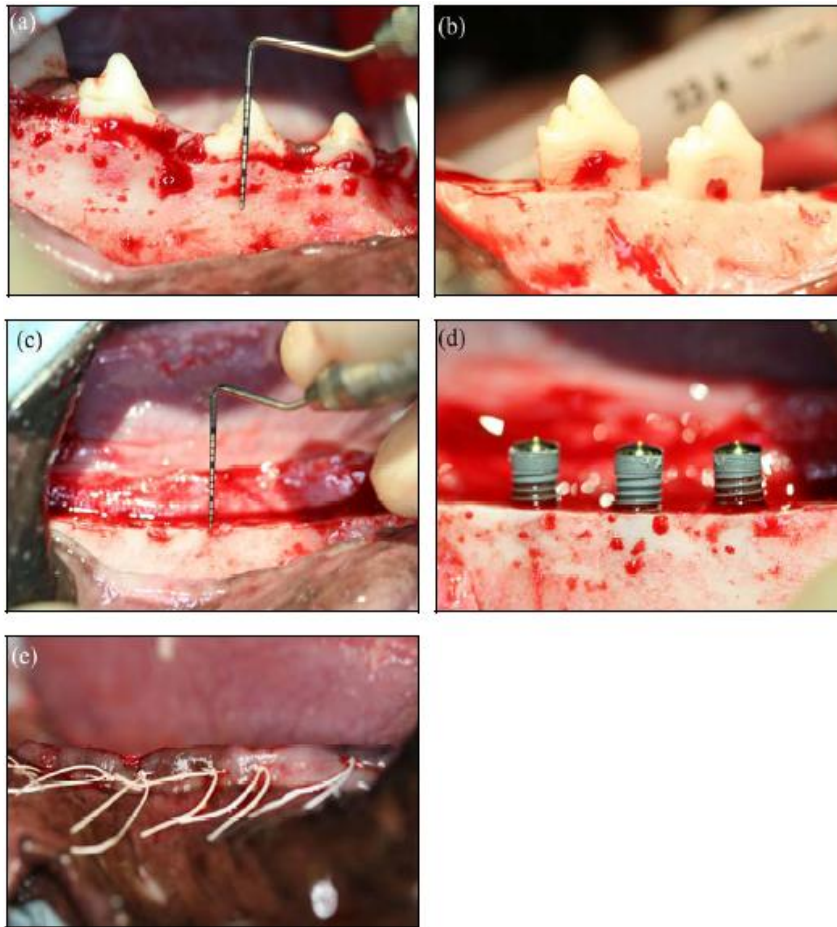


Figure 9.1. (a) Buccal and lingual mucoperiosteal flaps reflected. The probe is used to determine the approximately 6 mm alveolar reduction from the cemento-enamel junction of the mandibular premolar teeth. (b) Alveolar bone is circumferentially reduced using water-cooled rotating burs. The premolar teeth are then extracted and the 1st molar amputated at the level of the reduced alveolar crest. (c) The alveolar process is carefully planed using large water-cooled rotating burs to create a plane facilitating precision dental implant placement. (d) $\varnothing$ 4.0-4.3/10-mm dental implants are placed into the extraction sites of the distal root of the 3rd premolar, and the mesial and distal root of the 4th premolar in each jaw quadrant. Five mm of the implant is placed within the surgically reduced alveolar crest, leaving 5 mm of the implant above the crestal plane. (e) Coronally advanced tension-free flaps submerge the implants and any implanted device, biomaterial or biologic construct. The flaps are advanced and adapted 3–4 mm coronal to the implants followed by wound closure using expanded polytetrafluoroethylene/ ePTFE sutures and mattress suturing techniques.

The maxillary 1st, 2nd, and 3rd premolar teeth are surgically extracted and the maxillary 4th premolars reduced in height in order to alleviate potential trauma from the maxillary teeth to the experimental mandibular sites. Experienced surgeons perform all procedures.

9.8.2. Postsurgery Protocol

A long-acting opioid (buprenorphine HCl; 0.01-0.03 mg/kg IM, BID/3days) and a broad-spectrum antibiotic (enrofloxacin; 2.5 mg/kg IM) are administered postsurgery. Plaque

control is maintained by daily flushing the oral cavity with chlorhexidine gluconate (20-30 ml of a 2% solution). Observations of experimental sites with regards to gingival health, maintenance of suture line closure, edema, and evidence of tissue necrosis or infection are recorded daily. Sutures are removed at approximately 10 days postsurgery.

9.8.3. Radiographic Recordings and Analysis

Radiographs are obtained under sedation immediately postsurgery, and at 4 and 8 weeks postsurgery (telazol 5 mg/kg - xylazine 1 mg/kg IM) using a mobile x-ray unit (Aribex™ Nomad™, Aribex Inc, UT, USA) and a standardized protocol including an intraoral digital sensor (Kodak RVG 6000 Digital Radiography System, Eastman Kodak Company, Rochester, NY, USA) aligned with a laptop computer for parallel technique image acquisition and storage.

One masked experienced examiner evaluates the computer enhanced radiographic images. The following recordings are made for each implant:

- *Bone regeneration* (vertical augmentation of the alveolar ridge): distance from the 5-mm thread to the coronal extension of newly formed bone along the mesial and distal aspect of each implant.
- *Peri-implant bone remodeling*: an implant is scored positive for peri-implant bone remodeling when a radiolucent zone can be observed around the implant in resident bone at 4 and/or 8 weeks compared with immediately postsurgery.
- *Implant dislocation*: an implant is scored positive for dislocation when the implant has tipped, drifted, extruded, or rotated at 4 and/or 8 weeks compared with its position immediately postsurgery.
- *Seroma formation*: presence of a circular/ovoid peri-implant radiolucent zone in induced bone at week 4 and/or 8 is scored as seroma formation.

9.8.4. Fluorescent Bone Labeling

The animals optionally receive fluorescent bone labels [11] for a qualitative evaluation and temporal progression of bone formation. Oxytetracycline hydrochloride (Maxim-200, Phoenix Pharmaceuticals, St. Joseph, MO, USA; 25 mg/kg, SQ) is administered at 3 weeks, xylenol orange (Sigma-Aldrich Inc., St. Louis, MO, USA; 200 mg/mL; 90 mg/kg, SQ, twice one day apart) at 4 weeks, and calcein (Sigma-Aldrich Inc., St. Louis, MO, USA; 25 mg/mL; 5 mg/kg, SQ) at 10 and 3 days pre-euthanasia.

9.8.5. Euthanasia and Perfusion

The animals are euthanized at 8 weeks postsurgery. Briefly, they are anesthetized (see above) to allow placement of a 20/23-gauge intravenous catheter in the foreleg. Pentobarbital (30 mg/kg IV) is administered to effect until a deep plane of anesthesia is reached. Next, the

carotid artery and jugular vein are exposed and Tygon tubing inserted via a 20 G catheter into the carotid artery. Heparin (100 U/kg IV) is administered 5 min before euthanasia to slow coagulation. The animals are then euthanized using an intravenous injection of concentrated sodium pentobarbital (Euthasol®, Delmarva Laboratories, Inc, Midlothian, VA, USA), the jugular veins are severed, and a 0.9% saline/0.1% lidocaine/30,000U heparin flush initiated. The saline/lidocaine/heparin flush is continued until a clear outflow appears. The animals are then perfused using 9-10 L McDowell's fixative - 13.4 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2.7 g NaOH, 100 mL 37-40% formaldehyde, 20 mL 25% glutaraldehyde, 0.1% lidocaine. [53] Block sections including implants, bone and soft tissues in contralateral quadrants are collected using a conventional block biopsy protocol. The block specimens are rinsed in sterile saline and transferred to a volume of McDowell's fixative 10 times that of the block sections prior to histotechnical processing.

9.8.6. Histotechnical Processing

The tissue blocks are fixed in McDowell's fixative for 3-5 days, dehydrated in alcohol, and embedded in methylmethacrylate resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany). The implants are cut mid-axially in a coronal-caudal plane into 200- μm thick sections using the cutting-grinding technique (EXAKT Apparatebau, Norderstedt, Germany), and subsequently ground and polished to a final thickness of approximately 40 μm . [54,55] Unstained central sections are used for fluorescent light microscopy analysis and imaging. The same selected sections are then stained using Stevenel's blue and van Gieson's picro fuchsin for routine histopathologic and histometric analysis using incandescent and polarized light microscopy (Figure 9-2).

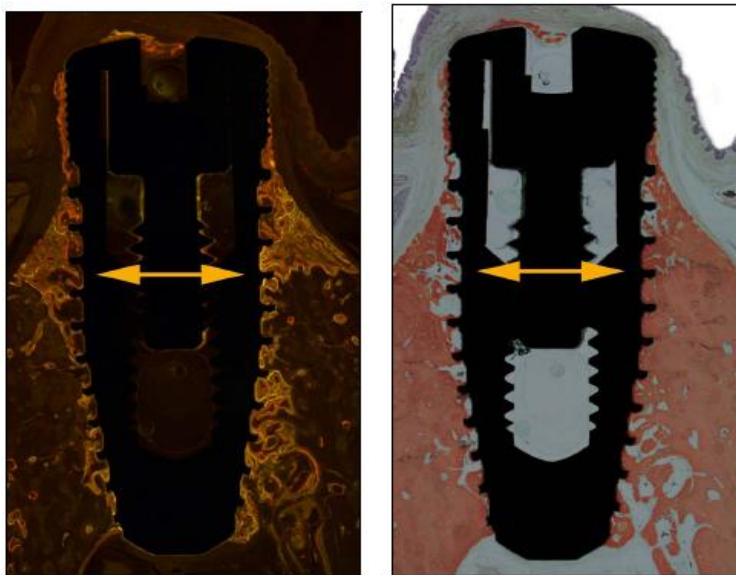


Figure 9.2. Unstained and stained (Stevenel's blue and van Gieson's picro fuchsin) central sections are used for the fluorescent (left) and incandescent light (right) microscopy analysis. Note new bone formation approaching the implant platform.

9.8.7. Histologic Analysis

One calibrated masked examiner performs the analysis of the fluorochrome bone histodynamic markers using fluorescent light microscopy. Bone formation is observed relative to the presence or absence, intensity, and width of the fluorochrome markers at the 3-, and 4-week and pre-sacrifice observations (evidenced by yellow, orange, or green labels, respectively).

The histologic analysis using incandescent and polarized light microscopy includes observations of peri-implant bone formation and remodeling (formation/resorption), woven/lamellar bone, fibrovascular tissue and marrow, seroma formation, inflammatory responses, and vascularity.

9.8.8. Histometric Analysis

A calibrated masked examiner performs the histometric analysis. The following measurements are recorded for the buccal and lingual surfaces of each implant (Figure 9–3):

- *Bone Regeneration (height)*: distance between the 5-mm thread and the vertical extension of newly formed bone along the implant. Bone formation exceeding the implant platform is not included.
- *Bone Regeneration (area)*: area of newly formed bone along the implant above the 5-mm thread. Bone formation exceeding the implant platform is not included.
- *Bone Density (new bone)*: ratio bone/marrow spaces in newly formed bone.
- *Osseointegration (new bone)*: percent bone-implant contact / BIC measured between the 5-mm thread and the vertical extension of newly formed bone along the implant.
- *Bone Density Outside the Implant Threads / BD_{OT} (resident bone)*: ratio bone/marrow spaces in a 400 x 4000 μm area (width x height) immediately outside the implant threads in resident bone.
- *Bone Density Within the Implant Threads / BD_{WT} (resident bone)*: ratio bone/marrow spaces within the implant threads in resident bone.
- *Osseointegration (resident bone)*: percent BIC within resident bone measured from the 5-mm thread to the apex of the implant.

9.8.9. Outcomes

The critical-size supraalveolar peri-implant defect model has been used to evaluate devices, biomaterials and biologic constructs with special emphasis on BMPs. The clinical relevance of this model is readily realized from its defect morphology and dimensions (height 5 mm/width 5-6 mm/volume ~ 3 cc) similar to challenging human alveolar defects allowing use of off-the-shelf devices, biomaterials and implants specified for human use.

These characteristics also allow clinicians an immediate and objective assertion of how observations in this model translate to real life applications.

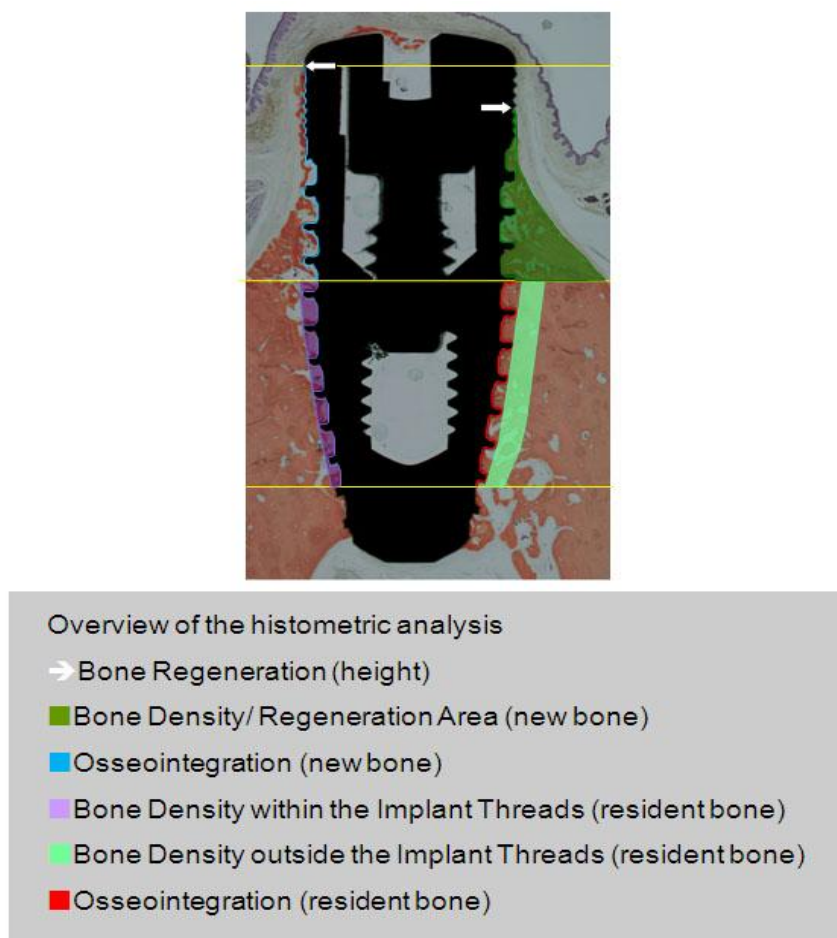


Figure 9.3. Histometric parameters.

For example, split-mouth comparisons clearly demonstrate a clinically relevant potential of an rhBMP-2/ACS construct to support significant vertical augmentation of the alveolar process. [45] As expected, minimal bone formation occurred in contralateral jaw quadrants receiving the ACS carrier alone indicating the limited potential of this preclinical model for self-regeneration (mean vertical bone formation: rhBMP-2/ACS: 4.2 mm vs. ACS: 0.5 mm). The combination of the rhBMP-2/ACS construct with space-providing devices predictably induced bone formation that not only was significantly superior to available technologies, but also yielded clinically relevant ridge-form and volume. [41,42] Thus, the critical-size supraalveolar peri-implant defect model allows a biological evaluation of new technologies in a relevant milieu, as well as engineering of clinically relevant tissue dimensions, as shown evaluating the application of rhBMP-2 in a thermo-dynamic calcium phosphate putty — α -BSM®. [43]

The critical-size supraalveolar peri-implant defect model also provides opportunity to evaluate *in vivo* dynamics of biomaterials used as carriers for biologic agents or as stand-alone treatments. For example, in contrast to robust bone formation in response to the rhBMP-2 constructs described above, surgical implantation of DFDBA (lyophilized demineralized bone matrix) combined with a space-providing guided bone regeneration

membrane resulted in approximately 3-fold less bone formation (mean vertical bone formation: 1.5 mm) indicating the limited potential, if any, for this not unusual clinical treatment combination to support osteogenic bone formation. [29] Rather, the DFDBA particles were found dispersed in a dense connective tissue matrix and in close contact to the titanium implant surface without any evidence of bone metabolic activity. The addition of rhBMP-2 to the DFDBA implant dramatically improved bone formation. [46].

Besides histological analysis of new bone formation, bone density, and osseointegration, the critical-size supraalveolar peri-implant defect model also allows temporal radiographic assessments of vertical bone formation and aberrant events including seroma formation, and other effects of implanted devices, biomaterials, and biologic constructs; assessment of dynamic events that may not be observable in the histologic evaluation.

Conclusion

Bone augmentation procedures have become increasingly important aspect of implant dentistry. Discriminating defect models applied in suitable animal platforms to evaluate the potential of candidate biomaterials or biologic constructs for alveolar reconstruction prior to clinical application are critical. Alveolar defect models can be basically divided into two categories: inlay and onlay defects. Inlay defects, due to their high innate regenerative potential, have shown limited value to discriminate therapies for alveolar bone augmentation. On the other hand, onlay models appear adequate tools for this purpose. Ideally, a candidate therapy should first be evaluated for biologic safety and potential using small animal platforms and screening models, such as the critical-size through-and-through rat calvarial osteotomy defect. Therapies that show biological potential in such qualified screening platforms/models should next be evaluated for efficacy and clinical potential in discriminating large animal critical-size models. Therefore, the animal may be considered a platform where the new therapy is tested, while the defect represents the actual model to evaluate the osteoinductive/ osteoinductive potential of the candidate technology. The critical-size, supraalveolar, peri-implant, defect model represents one such model that successfully has been used to select clinically relevant therapies for alveolar augmentation/ osseointegration.

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Chapter X

Animal Models for Sinus Augmentation

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10.1. Introduction

The choice of augmentation protocol appears dependent on the height of the residual alveolar process but also, more likely, clinician experience and preferences. The osteotome protocol entails transalveolar access to the subantral floor using a directional narrow-diameter spiral drill osteotomy incrementally widened while compacting the osteotomy walls through tapping using gradually increasing-diameter osteotomes. [1-7] The Schneiderian membrane becomes elevated following careful implosion of the subantral floor; and at the preference of the clinician, autograft bone, cadaver-sourced or synthetic biomaterials alone or in combinations are implanted to augment the immediate implant site. This technique can be applied with simultaneous placement of implants when more than 5-6 mm of residual bone is present and an increase of about 3 to 4 mm is expected. In cases of advanced alveolar atrophy and pneumatization, a lateral wall access osteotomy appears a preferred protocol to elevate the Schneiderian membrane and gain access for augmentation of the subantral floor. [8] This protocol, also known as the modified Caldwell-Luc procedure, was developed to obtain a larger space between Schneiderian membrane and subantral floor for bone augmentation to place dental implants. Thus the modified Caldwell-Luc procedure supports a significantly larger increase in bone volume than the osteotome technique; however without necessarily producing meaningful differences in alveolar support or osseointegration.

Even though there are reports pointing to a striking native potential for bone regeneration and osseointegration following elevation of the Schneiderian membrane without adjunctive implantation of autogenous bone or bone biomaterials, [9,10] maxillary sinus floor elevation

procedures commonly include the use of autologous bone grafts, allogeneic or xenogeneic bone biomaterials, synthetic biomaterials, biologic constructs, or combinations thereof at the discretion of the clinician. [11] An ideal graft, biomaterial, or biologic construct for alveolar augmentation also including subantral augmentation should 1) be osteoinductive (induce new bone formation) and/or osteoconductive (enhance osteogenic bone formation); 2) define and provide initial stability of the regenerative space; 3) allow convenient application (ease-of-use) for onlay and inlay indications; and 4) be bioresorbable while minimally compromising bone formation. [12,13] Importantly, newly formed bone must interact (osseointegrate) with immediate or delayed dental implants. Thus, before a novel technology for the sinus floor elevation/augmentation indication is introduced to the clinic, careful evaluation in well-characterized large animal model(s) should be carried out to confirm that the technology meets these essential criteria to allow dental implant placement, fixation and long-term retention.

10.2. Animal Platforms, Sinus Models and Outcomes

In sinus augmentation research, the animal should be regarded as a platform in which different approaches to sinus augmentation can be evaluated relative to biological principles and clinical application. A variety of platforms has thus been proposed and used to study sinus floor elevation procedures to include rabbits, dogs, sheep, goats, mini-pigs, domestic pigs, and nonhuman primates. Two main experimental approaches have been used depending on the location of implant placement. Dental implants can be placed through the alveolar process similar to the clinical application or through the sinus wall. For both approaches, access to the antral/subantral space can be gained creating a bone window in the lateral sinus wall and elevating the Schneiderian membrane. Whereas the first model closely resembles the clinic, it is also faulted by several practical inconveniences including the need to extract maxillary posterior teeth and a wait subsequent healing, variable crestal height, and limited intraoral surgical access for some species. Nevertheless, dental implant placement to evaluate osseointegration is essential to ensure the clinical validity of the augmentation protocol.

One must clearly define the research objective(s) prior to selecting an animal platform to be used. For studies investigating bone-implant interactions or evaluating bone biomaterials or biologics in various combinations, an understanding of the species-specific characteristics to include bone microstructure, composition, physiology, remodeling, and regenerative potential appear particularly important, [14] as well as consideration of characteristics analogous to humans and potential to produce conclusive data within an acceptable time frame. [15-17] Other important considerations include cost and care for animals, availability, acceptability to society, and ease of housing and handling. [15] Differences between animal platforms and the human subantral space relative to morphologic/dimensional characteristics such as proximity of sinus walls and relationship between the maxillary sinus and the alveolar ridge may complicate translation of preclinical observations to the clinical application. Further, the appositional bone formation rate may vary between species. Taken together, animal platform selection appears a critical step in the study of bone-inducing agents for

subantral augmentation and dental implant osseointegration; no single animal platform will be appropriate for all purposes. [14].

10.2.1. Rats

The rat maxillary sinus appears too small for relevant elevation of the sinus membrane and application of clinical bone augmentation protocols as well as dental implants. Thus the rat is not a realistic platform to study the sinus augmentation indication. [18].

10.2.2. Rabbits

The rabbit is one commonly used platform in biomedical research mainly due to its ease of handling and short time to reach maturity at around 6 months. [19,20] In sinus augmentation research, rabbits have been used less frequently than other animal platforms. [21] Rabbits feature a maxillary sinus with a well-defined ostium similar to that in humans, however its volume is a major limitation. As elevation heights up to 10 mm might be considered to mimic clinical settings in the atrophic human maxilla, the mean sinus volume in rabbits appears restricted — in the frequently used Japanese white rabbit approximating 2 mL. [22] An extra-oral approach to the antral space is necessary in the rabbit. In brief, a bone window is created in the nasal bone after the reflection of a skin flap. The Schneiderian membrane is elevated to allow the placement of the biologic construct being evaluated. [22-25] Small dimension titanium screws (“implants”) must be used and placed posterior to the osteotomy window to evaluate osseointegration. [25] In brief, biological and dimensional characteristics qualify the rabbit as a screening platform to evaluate candidate sinus augmentation protocols, however, large animal discriminating platforms/models are critical for definitive preclinical evaluations.

10.2.3. Dogs

The dog is one frequently used large animal platform in dental research with focus on alveolar bone and implant dentistry. [12, 26-30] However, in spite of similarities between humans and dogs with regards to bone characteristics and physiology, [31,32] only few studies have used the dog as a platform for sinus augmentation [21] possibly due to peculiarities in the anatomical structure of the maxillary sinus and composition of the Schneiderian membrane. [33] Thus the dog does not appear a preferred platform to evaluate candidate protocols for sinus augmentation.

10.2.4. Sheep and Goats

Sheep and goats are frequently used large animal platforms in orthopedic research. [34] They are easily obtained, bred, exhibit minimal genetic variation, and have suitable size for

maxillary sinus floor augmentation and are considered cost-effective platforms to study sinus augmentation. [35] Sheep have been used widely to study maxillary sinus augmentation; the adult sheep features maxillary sinus dimensions advantageous for sinus augmentation. [36] In general, sheep bones closely represent human bones macroscopically, even though the bone structure is quite different. [14] Sheep has a predominantly primary bone structure in comparison with the largely secondary human bone structure. Bone density in sheep is also significantly greater than in humans. [16] However, the sheep sinus still appears a valuable model relative to bone turnover and remodeling. [34,37-40] The goat also appears a suitable platform for testing clinical implants and materials as goats are considered to have metabolic and bone remodeling rates similar to that of humans. [41]

There are several reports concerning maxillary sinus augmentation using sheep and goats. [21,36,38,42-46] None has used immediate transalveolar dental implants due to the width/height of the alveolar bone makes it difficult to place an implant reaching into sinus. [35] Also, the Schneiderian membrane may in some breeds be considerably thicker than in humans. [45] Similar to rabbits, an extra-oral approach must be used to access the antral space of the maxillary sinus in sheep and goats since a lateral wall approach would demand additional surgical preparation including a lip incision to reach the first molar area. [36] Thus, a skin flap is used to access the lateral wall of the maxillary sinus. A bone window is created using burs or osteotomies, and the Schneiderian membrane elevated to create space for sinus augmentation. Two dental implants are placed either distal to or one on each side of the bone window. [37,38,44,46,47].

10.2.5. Pigs

The pig is considered to closely represent human bone anatomy, morphology and physiology, and thus has become a most common platform for subantral augmentation. [21, 48-55] The pig has also been used as a platform to evaluate new dental implant designs. [56-58] Porcine and human bone feature similarities in mineral density and concentration, [31] bone remodeling processes as well as lamellar structure, [59,60] and comparatively similar rate of bone regeneration and cortical bone mineralization. [61,62].

Domestic pigs are considered undesirable platforms due to their rapid growth and excessive final body weight. The development of the mini-pig has overcome this dilemma. The mini-pig platform has been shown to have several advantages. The mucosal lining of the mini-pig sinus is thin and vulnerable like in the human sinus. [48] The shortcoming of a mini-pig model is the insertion of the implants from the lateral sinus wall, because the infraorbital nerve is interposed between the alveolar process and the maxillary sinus [48] and the distance between the alveolar crest and the maxillary sinus floor does not allow a transalveolar approach. [36] The structure of the lateral wall of the maxillary sinus includes an outer *Substantia compacta*, an intermediate *Substantia spongiosa*, and an internal *Substantia compacta*. The mean width of the lateral wall approximates 3.0 mm similar to that of the severely atrophic human maxillary alveolar ridge. The maxillary sinus lateral wall represents an osteogenic potential similar to that of the sinus floor. [63] Because the maxillary sinus develops and continues to grow until the complete eruption of permanent teeth, mature animals must be used. [64] Although the thickness of the Schneiderian membrane in mature pigs averages 1.7 mm (0.6–1.3 mm in humans), the size and structure appears comparable to

the human with a *Stratum vasculare* and *Stratum reticulare*. [63,65] The access to the lateral wall of the maxillary sinus using an oral approach is complex, thus the extra-oral approach is preferred. [48] Shortcomings for this animal platform include cost, animal management and low cooperation.

10.2.6. Nonhuman Primates

Larger nonhuman primates closely relate to humans in size, anatomical structure, and bone metabolism. Nonhuman primates provide reliable prediction of biomaterials for sinus augmentation as the anatomy of the maxillary sinus and the appositional bone formation rate in primates closely parallels that in humans. [66,67] Macroscopic examinations of skulls of various primates demonstrate maxillary sinuses that are similar or larger than that of humans. [68] Maxillary sinus augmentation combined with clinically realistic dental implant placement using primate animal models has been shown. [10,69,70] Limitations for the use of nonhuman primates as a platform for research include high cost, potential for disease transmission, low cooperation, and importantly the animal's ability to manipulate and thus disturb a surgical site.

10.3. The Mini-Pig Sinus Augmentation Model

In considering size, bone physiology, handling and convenience of surgical approach, the mini-pig appears a preferred large animal platform to investigate subantral augmentation including bone-implant interactions, bone biomaterials and biologic constructs [48] described a surgical protocol adopted in a number of studies. [21,49-54,56,71,72] Our laboratory has used this protocol detailed below to evaluate the effect of bone morphogenetic proteins on sinus augmentation. [55].

10.3.1. Experimental Surgery

Food is withheld the night preceding surgery. The animals are pre-anesthetized with atropine (0.02-0.04 mg/kg IM), buprenorphine HCl (0.01-0.03 mg/kg IM), and acepromazine (0.2-0.3 mg/kg IM). After tranquilization, an IV catheter (18g) is placed in the foreleg for induction with propofol (5-7 mg/kg IV). Animals are then moved to the operating theatre and maintained on gas anesthesia (1-2% isoflurane/O₂ to effect). The animals receive a slow constant rate infusion of lactated Ringer's solution (10-20 ml/kg/hr IV) to maintain hydration intra-surgery. Depth of anesthesia is monitored by lack of response to toe pinch, lack of corneal reflex, and by monitoring the depth of respiration. Routine dental infiltration anesthesia (lidocaine HCl 2%, epinephrine 1:100,000) is used at the surgical sites.

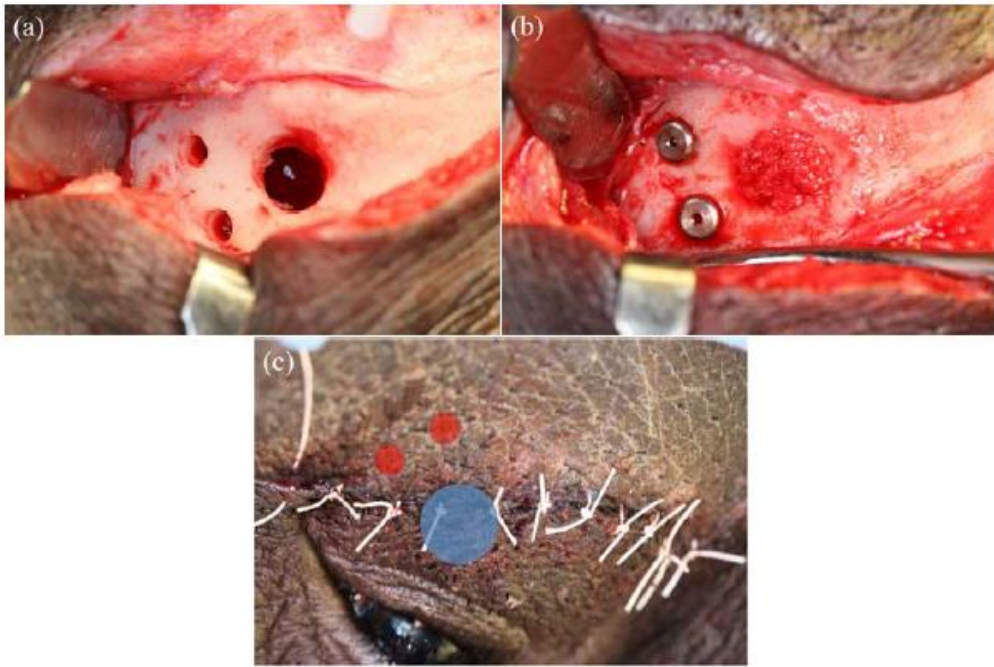


Figure 10.1. Surgical access to the sinus using the extraoral lateral approach. (a) Access preparation to elevate the Schneiderian membrane and osteotomy preparation for placement of two titanium dental implants; (b) Placement of bone graft and implants with cover screws; and (c) Wound closure in layers also showing the positioning of the access osteotomy and dental implants.

The sinus augmentation procedure uses an extra-oral approach (Figure 10–1). The initial incision is outlined using a surgical marker; a horizontal line parallel to the inferior orbital rim is drawn 7–10 mm below its edge extending to the lateral border of the nasal ridge. This line/markings is used as a guide for the initial incision, which will provide access to the surgical site. Briefly, through the infraorbital skin incision, a periosteal flap is raised to expose the anterior sinus wall, the zygomaticoalveolar crista and the malar prominence. An access osteotomy to the sinus, a 8–15 mm diameter bone window depending on animal breed and size, is prepared immediately below the malar prominence using piezosurgery, and the Schneiderian membrane is gently elevated avoiding perforation. Following creation of the bone window, routine dental implant osteotomies are performed. Two osteotomies are placed obliquely below bone window approximately 4–8 mm apart depending on animal breed and size. The sinus membrane must be protected during osteotomy preparations using a curved sinus elevator to avoid accidental perforations. The lateral sinus wall (cortical bone) increases in thickness posteriorly, which may affect the amount of resident bone used for primary stability of the implants. If the osteotomies are correctly positioned, the thickness of cortical wall at the site should approximate 3 mm sufficient to provide primary stability for the implants. Two dental implants are then positioned prior to placement of the candidate sinus augmentation technology. Implants that are 10 mm or greater in length are preferred to allow evaluation of relevant bone formation. After placement of the implants protruding into the sinus cavity with primary stability, about 4 mL of the augmentation technology may be used to fill the space underneath the elevated Schneiderian membrane through the access bone

window. The surgical site(s) is then closed and sutured in layers using resorbable and non-resorbable sutures as appropriate.

10.3.2. Postsurgery Protocol

A long-acting opioid (buprenorphine HCl, 0.01-0.03 mg/kg IM, BID/3 days) is administered for pain control. A broad-spectrum antibiotic (ceftiofur sodium; 3 mg/kg IM, SID/5 days) is administered for infection control. Sutures are removed under sedation (telazol 5 mg/kg - xylazine 1 mg/kg IV) at approximately 10 days postsurgery. The experimental sites are monitored daily until suture removal and thereafter at least weekly for swelling/dehiscencies/infection.

10.3.3. Fluorescent Bone Labeling

The animals optionally receive fluorescent bone labels [73] for a qualitative evaluation and evaluation of the temporal progression of bone formation. Oxytetracycline hydrochloride (200 mg/mL; 25 mg/kg, SQ) is administered at 3 weeks, xylenol orange (200 mg/mL; 90 mg/kg, SQ, twice one day apart) at 4 weeks, and calcein (25 mg/mL; 5 mg/kg, SQ) at 10 and 3 days pre-euthanasia.

10.3.4. Euthanasia and Perfusion

Our laboratory routinely uses an 8-week healing interval providing relevant bone formation and maturation at which time the animals are euthanized for histologic evaluation. The animals are anesthetized (see above) to allow placement of a cephalic vein catheter. Pentobarbital (30 mg/kg IV) is administered to effect until a deep plane of anesthesia is reached. Next, the carotid artery and jugular vein are exposed and Tygon tubing inserted via a 20 G catheter into the carotid artery. Heparin (100 U/kg IV) is administered 5 min before euthanasia to slow coagulation. The animals are then euthanized using an intravenous injection of concentrated sodium pentobarbital, the jugular veins are severed, and a 0.9% saline/0.1% lidocaine/30,000U heparin flush initiated. The saline/lidocaine/heparin flush is continued until the outflow appears clear. The animals are then perfused using 9-10 L McDowell's fixative (13.4 g NaH₂PO₄·H₂O, 2.7 g NaOH, 100 mL 37-40% formaldehyde, 20 mL 25% glutaraldehyde, 0.1% lidocaine. [74] Block sections including implants, bone and soft tissues in contralateral jaw quadrants are collected using a conventional block biopsy protocol. The block specimens are rinsed in sterile saline and transferred to a volume of McDowell's fixative 10 times that of the block sections prior to histotechnical processing.

10.3.5. Histotechnical Processing

The tissue blocks are fixed in McDowell's fixative for 3-5 days, dehydrated in alcohol, and embedded in methylmethacrylate resin (Technovit 7200 VLC, Kulzer, Wehrheim,

Germany). The implants are cut mid-axially in a coronal-caudal plane into 200 μm thick sections using the cutting-grinding technique (EXAKT Apparatebau, Norderstedt, Germany), and subsequently ground and polished to a final thickness of approximately 40 μm . [75,76] Unstained central sections are used for fluorescent light microscopy analysis and imaging. The same selected sections are then stained using Stevenel's blue and van Gieson's picro fuchsin stains for histopathologic and histometric analysis using incandescent and polarized light microscopy.

10.3.6. Histopathologic Analysis

The histopathologic analysis includes incandescent, polarized and fluorescent light microscopy. Bone formation is observed relative to the presence or absence, intensity, and width of the fluorochrome markers at the 3-, and 4-week and pre-sacrifice observations (evidenced by yellow, orange, or green labels, respectively) using unstained sections. Peri-implant bone formation and remodeling (formation/ resorption), woven/lamellar bone, fibrovascular tissue and marrow, seroma formation, inflammatory responses, and vascularity are observed using the same slides following staining.

10.3.7. Histometric Analysis

The histometric parameters recorded for the most central stained section(s) of each implant shown in Figure 10–2 include:

- *New bone height*: Distance from the internal aspect of the maxillary sinus cortical plate to the most apical aspect of newly formed bone along the implant long-axis including bone formation extending beyond the implant apex;
- *Residual bone height*: Distance from the external to the internal aspect of the maxillary sinus cortical plate along the implant long-axis;
- *Bone density outside the threads*: Fraction mineralized bone immediately outside the implant threads along the entire length of the implant in a 500- μm zone within the extension of newly formed and resident bone, respectively;
- *Bone density within the threads*: Fraction mineralized bone within the root of the implant threads within the extension of newly formed and resident bone, respectively; and
- *Osseointegration*: Fraction mineralized bone-implant contact (BIC) measured along the entire length of the implant within the extension of newly formed and resident bone, respectively.

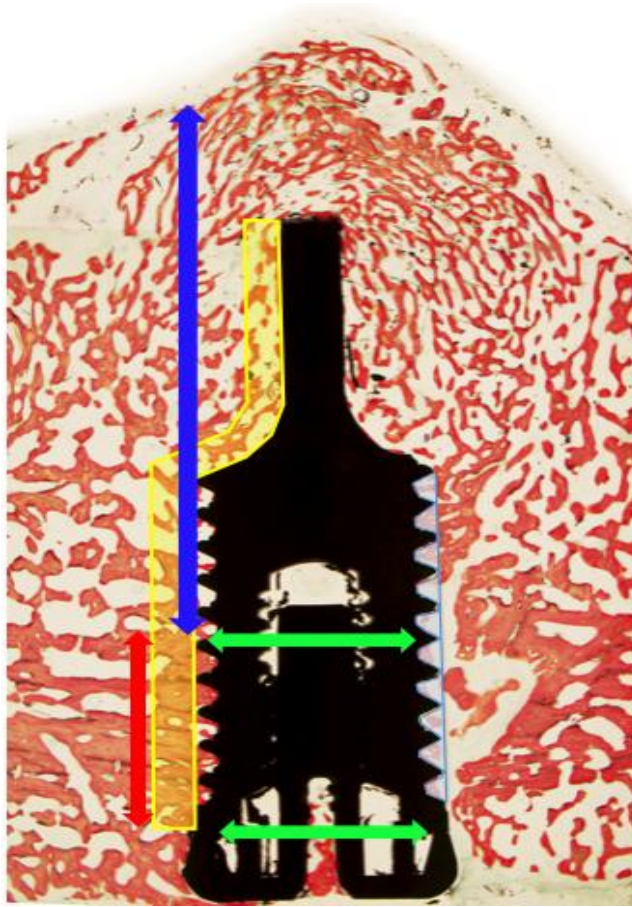


Figure 10.2. Histometric assessment: green arrows external and internal aspect of the sinus lateral wall; blue arrow; new bone height, red arrow; residual bone height (thickness of the sinus lateral wall), white box: bone density within the roots of the threads, yellow box; bone density immediately outside the titanium implant threads.

Conclusion

To overcome the deficient alveolar ridge in the posterior maxilla for implant treatment, sinus augmentation has to be considered. However, bone biomaterials or biologic constructs for sinus augmentation must first be evaluated in a well-characterized model prior to clinical introduction. Understanding advantages and limitations of various animal species to serve as platforms to study subantral augmentation thus becomes essential. While the rabbit qualifies as a screening platform to evaluate candidate protocols, definitive preclinical assessment must be performed using discriminating large animal platforms. The mini-pig maxillary sinus using a lateral extraoral approach thus appears widely preferred. Histopathologic and histometric analysis including bone fluorescent markers generate adequate data to project clinical efficacy. Dental implant placement is a critical aspect of the experimental protocol to confirm that the technology meets essential clinical expectations of implant osseointegration.

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Chapter XI

Ethics and Regulation in the Use of Laboratory Animals

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Abstract

The ethical and scientific questions raised by the use of laboratory animals in procedures which may cause them pain, suffering, distress and/or lasting harm, are discussed in terms of the Three Rs (*Reduction, Refinement, Replacement*) concept of Russell and Burch; the opportunities for replacement offered by new technologies; the laws which seek to protect animals whilst taking account of the legitimate demands of science, industry and human welfare; and the regulations which govern the manufacture and marketing of chemicals, medicines, medical devices in general, and dental implants in particular. It is concluded that, for both ethical and scientific reasons, there must be an ongoing and endless review of all the test procedures that are being developed, validated, accepted and applied — and, most of all, of what is demanded by the regulatory authorities. Meanwhile, the new technologies undoubtedly offer many exciting possibilities, but the challenge is to find intelligent pathways through their variety and complexity toward the goal of improving the protection of human health and the environment.

11.1. Introduction

Humans and animals have evolved in parallel for thousands of years, and this has resulted in a variety of relationships between them, overwhelmingly to the benefit of the humans.

Humans progressively came to use animals to provide them with food and clothing, to perform a variety of tasks, for entertainment, as companions, and, in more-recent times, as objects for study in invasive scientific experiments.

These relationships have been explored since ancient times, in cave paintings, fables, legends, and philosophies. Ultimately, it is the humans who have the power to control the animal–human relationships, and this has resulted in a wide spectrum of consequences in all these relationships, from care and compassion at one end to outright cruelty at the other. That man’s exploitation of animals is considered to be morally acceptable stems from the hierarchical concept of “dominion”, which implies ownership and the power to decide, coupled with the belief that animals are inferior to humans and are therefore less entitled to consideration.

Discussions about the moral status of animals have featured in many religions and philosophies, but it was the development of experimental animal physiology in the 19th century, principally as a means of increasing understanding of the human body, and of diseases and how they could be treated, which brought the animal experimentation issue to the fore.

The problem is the extent to which it should be permissible to subject laboratory animals to procedures which might cause them pain, suffering, distress and/or lasting harm. The dilemma is that, whereas in other situations, such as farm animal husbandry, reasonable compromises between animal welfare and animal use are possible, many of the procedures applied to laboratory animals will inevitably and unavoidably cause significant suffering.

Not surprisingly, there exists a wide spectrum of views on this issue. At one extreme, there are the animal rights activists, some of whom are prepared to commit criminal and violent acts in pursuance of their ambition to end all animal experimentation. At the other extreme are the libertarians, who assert that virtually anything is permissible. Most people occupy positions at different places across the spectrum between these extremes, and this involves making decisions, not only about *whether* animal experimentation should be permitted, but *what* should be permitted, under *what circumstances*, and how it should be regulated.

Experiments are conducted on living animals (by which we mean living vertebrates) for three main reasons: education, research and testing. It is very difficult to justify the causation of suffering in the name of education, even in the training doctors, dentists and veterinarians, and in research, it is advisable to minimise suffering, in the interests of the science, as well as the animals.

It is in the induction of diseases in animals as models for humans, and in the testing of chemicals, medicines, medical devices, vaccines, pesticides, and various other products, that the causation of pain and suffering may be unavoidable.

In this chapter, we will discuss the ethical issues raised by animal experimentation, the solution to the moral dilemma offered by the Three Rs (*Reduction, Refinement, Replacement*) concept of Russell and Burch, [1] some of the laws which control animal experimentation, the testing required by regulations of various kinds, and the specific question of dental implant testing. While we recognise that the use of some *invertebrate* animals raises questions about pain and suffering, as a result of which they are included in some of the laws and regulations which govern animal experimentation, our discussion will be restricted to *vertebrate* animals.

11.2. Ethical Issues

It is impossible to cover the wide-ranging and diverse ethical and philosophical views associated with the animal experimentation question in this single chapter. Instead, our aim is to give the reader an overview of the key ethical perspectives.

How experiments on animals are perceived, and for scientists, how they are conducted, depends on the underlying ethical viewpoints on the use of animals. While there are many strongly held and intertwined beliefs about animal experiments, and a great deal of literature has been devoted to discussing them, e.g. [2-5] there are three overarching standpoints that seem to emerge repeatedly.

Each of them is expressed here in terms of rights, and more specifically, the right to moral consideration. From this general overview alone, the reader will appreciate the complex nature of ethics, and will see how the same theory can be used to support diametrically-opposed views.

11.2.1. Animals Should Have Equal Rights

That animals have their own intrinsic value, and the right to moral consideration equal to that given to humans, is the stance taken by those who want to have animal experimentation abolished immediately. In essence, they feel that humans have no right to inflict pain or harm on any other animal, no matter what steps are taken to alleviate that harm, and regardless of the benefits that might result. Perhaps the most widely quoted philosopher to support this stance was Jeremy Bentham, who is seen as one of the founders of the ethical theory of utilitarianism. He proposed that the interests of every being affected by an action should be taken in to account and given the same weight as the interests of any other being. He felt that all beings have a right to equal consideration, and pointed to the capacity to suffer as the vital criterion, in his now famous quote:

The question is not, can they reason?, nor, can they talk?, but, can they suffer? [6]

Today, one of the most well-known protagonists for giving equal consideration to animals is Peter Singer, who uses this principle to argue against speciesism toward animals, as a form of prejudice analogous to racism:

Racists violate the principle of equality by giving greater weight to the interests of members of their own race, when there is a clash between their interests and the interests of another race ... Similarly, speciesists allow the interests of their own species to override the greater interests of members of other species. [7]

Thus, he says, if we do not, as we should not, accept racism, then we should also not accept that the suffering of animals is less important than the suffering of humans, so we should not use them in experiments likely to cause them harm.

11.2.2. Animals Should Have No Rights

In polar opposition to the intrinsic value argument, is the contention that the superiority of humans grants them free reign to use other animals as they wish. Thus, all animal use for the benefit of humans is legitimate, whatever the cost to the animals themselves — so, in essence, animals have no right to moral consideration. The writings of the philosopher, Immanuel Kant, are often used to support this stance. His concepts were deontological in nature, that is, they were related to the notion of duty and principle. He argued that:

So far as animals are concerned, we have no direct duties. Animals are not self-conscious and are there merely as a means to an end. That end is man. Our duties towards animals are merely indirect duties towards humanity. [8]

Thus, the moral consideration should not be given to the animals, but to the humans, in that any actions to prevent needless suffering for animals are grounded in the advantages for humans, such as not fostering cruelty. This is illustrated by Kant's view on animal experiments:

Vivisectionists, who use living animals for their experiments, certainly act cruelly, although their aim is praiseworthy and they can justify their cruelty, since animals must be regarded as man's instruments; but any such cruelty for sport cannot be justified. [8]

It is clear that these equal rights/no rights standpoints can never be reconciled. They are at opposite ends of the ethical spectrum and have been open to thorough analysis, criticism and discussion. e.g. [4, 9].

11.2.3. The Continuum of Rights

The ethical issues are often considered in terms of a scale of rights, whereby different types of animal should be given different degrees of consideration, because of their different degrees of sentience, linked to the justification of usage on the basis of cost–benefit analysis. Thus, since humans, themselves being animals, have greater intrinsic value than other animals, the use of other animals for the benefit of humans, as well as the environment and yet other animals, can be justified, provided that the benefits to humans sufficiently outweigh the costs to the animals used. The overall implication is that it is morally wrong to use non-human animals for purposes which cannot be justified, or to cause unnecessary or avoidable suffering to them.

This principle is utilitarian in nature, *the greatest good for the greatest number* being the key factor. It provides an ethical justification for deciding on the goodness or badness of an act by reference to its consequences in the context of given circumstances. However, in practical terms, it does not embrace the all-things-are-equal aspect of utilitarianism, as animals are often not given equal consideration during the cost–benefit analysis detailed below, which tends to be biased in favour of humans.

11.2.4. The Role of Ethics in Legislation

The continuum of rights principle reflects the majority view among the general public, and forms the basis, for example, of the European legislation on animal experiments and of the cost–benefit analysis which is required by that legislation. This cost–benefit analysis is a difficult balancing act to apply in determining whether or not an animal experiment can be justified.

The costs are to the animals in terms of pain, suffering distress or lasting harm, and have to take into account the capacity of the animals concerned to be aware of what they might endure, and the number of them that will be likely to endure it. The benefits are usually to humans, but may also be to the environment or other animals, and their analysis needs to:

- include an objective assessment of the value of the potential benefits;
- take into account the quality, validity and necessity of the proposed methods in achieving the potential benefits; and
- involve an overall assessment of the project, in order to judge the likelihood that those potential benefits will be realised, given the proposed scientific approach.

Various scoring systems have been proposed, in attempts to make this decision-making more objective, such as the Decision Cube, Ethical Scores, Checklists, and the Extended Animal Welfare Assessment Grid. [1, 10].

However, it is inherently difficult to be completely unbiased, because, in practice, the costs to the animals are not given the same consideration/weight as are the benefits to humans. There is also a great amount of debate about whether certain species, such as non-human primates (hereafter called primates), which are known for their high level of sentience and social and behavioural complexity, should be given more consideration than other species, such as mice and rats. [4].

11.2.5. Conclusion

While it may be difficult to decipher which stance is the most appropriate, it is important to recognise and apply ethical considerations in dealing with the problem of how, when and if, to legislate on animal experimentation. Indeed, not only are ethics enshrined within much of the legislation, they are also explicitly considered through voluntary, and in some cases, mandatory, ethical bodies and/or processes within establishments, when animal research is being proposed.

In the UK, an Ethical Review Process (ERP) has been introduced, to ensure that all the establishments in which animals are used will foster an appropriate culture of care. The ERP plays an invaluable role in ensuring the proper justification of animal experiments, and in encouraging the implementation and dissemination of best practice with regard to both the scientific and animal welfare aspects of a proposed project. [11].

11.3. Reduction, Refinement, and Replacement

11.3.1. The Principles of Humane Experimental Technique

In 1954, concerned by the surge in animal experimentation in the UK which had followed the Second World War, the Universities Federation for Animal Welfare (UFAW) decided to conduct a systematic study on the application of humane experimental techniques to laboratory animals, and appointed a young zoologist, William Russell, and a young microbiologist, Rex Burch, to work on the project. In 1957, at a UFAW-sponsored *Symposium on Humane Technique in the Laboratory* that took place at Birkbeck College, London, Russell discussed the progress being made in the study, and introduced what is now known as the Three Rs concept, in a lecture entitled *Modes of Increasing Humanity: Replacement, Reduction and Refinement*. [12] Two years later, Russell and Burch's outstanding book, *The Principles of Humane Experimental Technique*, was published. [1].

The main principle on which Russell and Burch based their analysis is that:

It is widely recognised that the humanest possible treatment of experimental animals, far from being an obstacle, is actually a prerequisite for successful animal experiments.

They considered the central problem to be:

That of determining what is and what is not humane, and how humanity can be promoted without prejudice to scientific and medical aims.

They began with a discussion of *the concept of inhumanity and its relation to those of pain and distress*, then turned to *the positive aspect – the analysis of methods of diminishing inhumanity* when performing laboratory animal experiments. They said that:

We must first distinguish direct and contingent inhumanity. By the former, we mean the infliction of distress as an unavoidable consequence of the procedure employed. By the latter, we mean the infliction of distress as an incidental and inadvertent by-product of the use of the procedure, which is not necessary for its success.

Their main thesis was that:

Inhumanity can be, and is being, diminished or removed under the three broad headings of Replacement, Reduction, and Refinement – the Three Rs of humane technique:

- Reduction means reduction in the numbers of animals used to obtain information of a given amount and precision.
- Refinement means any decrease in the incidence or severity of inhumane procedures.
- Replacement means the substitution of conscious living higher animals by insentient material.

They added that:

Replacement is always a satisfactory answer, but reduction and refinement should, wherever possible, be used in combination.

Although warmly welcomed at the time, the Three Rs concept was more or less ignored during the 1960s. However, the 1970s saw renewed interest, notably in the UK, as a result of a campaign to mark the centenary of the *Cruelty to Animals Act 1876*, which was still the basis for regulating animal experimentation in 1975. Then, in 1978, having conducted a survey for the Research Defence Society, David Smyth gave us the Three Rs definition of alternatives, to include:

All procedures which can completely replace the need for animal experiments, reduce the number of animals required, or diminish the amount of pain or distress suffered by animals in meeting the essential needs of man and other animals. [13]

This very useful definition is more than a mere restatement of the Three Rs, since it imposes on all those who propose to use laboratory animals, the burden of providing convincing arguments that their work is necessary for some good purpose. This requirement for scientific and ethical justification and the application of the Three Rs is now explicit in a number of national and international laws on the protection of laboratory animals, as well as in many of the regulations which govern the testing of chemicals, medicines, medical devices, and other chemical products.

11.3.2. Reduction

In essence, *reduction* is about reducing the numbers of animals used in research without losing valuable information. The concept has evolved since its inception in 1959, and it can now be defined as:

Obtaining comparable levels of information from the use of fewer experimental animals, or of obtaining more information from a given number of animals, so that fewer animals are needed to complete a given research project, taking into account individual animal welfare in relation to minimising pain, suffering, distress or lasting harm. [14]

Russell and Burch felt that:

Reduction remains of great importance and of all modes of progress it is one most obviously, immediately, and universally advantageous in terms of efficiency. [1]

They suggested three main ways in which *reduction* could be achieved, via: a) better research strategy; b) better statistical analysis; and c) better control of variation. Unfortunately, there has been, and still is, confusion about how to address and implement these considerations. Indeed, the first two of them perhaps provide the greatest opportunity to reduce animal use immediately and with very little investment of effort, yet awareness about efficient experimental design and the appropriate use of statistical methods still remain extremely low among biomedical scientists.

11.3.2.1. Controlling Variation

Opinion on the control of variation is divided, particularly among toxicologists. Russell and Burch noted that the accuracy of an experiment:

Depends on the size of sample, the extent to which individuals of the species vary in response to the drug, and the efficiency in design and analysis of our experiment. [1]

This variance can be genetic and non-genetic. The argument goes that, because humans have different sensitivities to substances and so vary in their responses, then a heterogeneous, i.e. genetically variable, group of animals must be used, to also provide a wide range of responses. However, though Russell and Burch showed that it is better to control and identify this genetic variation in the animals, this fact is still not appreciated and implemented in most cases, and in particular, toxicologists remain very reluctant to use inbred strains, i.e. groups of genetically identical animals. In a recent editorial, Festing provided an excellent and modern overview of why this is so important, and also explained Russell and Burch's high fidelity fallacy, which is also applicable to other areas of biomedical research. [15].

Fidelity is the extent to which a model resembles the target in every respect. A cell culture is a low fidelity model, because it is very unlike an intact human, whereas another primate is a high fidelity model, because it is very similar to a human. The fallacy is the assumption that models always need to be high fidelity models and that all high fidelity models are useful. As Festing explains, what are actually needed in toxicity testing are models that can discriminate, e.g. between compounds which are toxic and non-toxic. The fidelity of the model, be it a cell or a primate, is not important, if it can be relied on to be discriminating in correctly predicting toxicity to humans. He concludes that we do not want models to be like humans in every respect, so we should certainly not attempt to model the uncontrolled genetic variation found in human populations. [15].

11.3.2.2. New Technologies

The application of new technologies and techniques can reduce the number of animals needed to achieve a given purpose. For example, in recent years, there has been a gradual increase in the application of non-invasive imaging techniques, such as the use of fluorescent markers and magnetic resonance imaging (MRI), which were originally developed and used in fundamental research or in medicine. As well as the obvious refinements that these techniques afford, they also allow diseases and responses to exogenous substances to be monitored in a temporal and spatial manner. This means that the sequential sacrificing of animals is not needed, as an individual animal can be studied throughout the duration of a study. Hence, a greater amount of information can be derived from a smaller number of animals. This can also increase the statistical validity of the data, by reducing the level of experimental variation. [16]

11.3.2.3. Using Animal Tissues

When scientists are considering conducting animal research, they should investigate whether they can still meet the aims of their study by replacing live animals with techniques that use cells or tissues from the same species (or whether they can alter their aims, in order to do so). Obviously, since many cell and tissue samples can be derived from each animal, the tissues from one animal could be used in a number of different projects. Some animals will

still need to be sacrificed, but the overall numbers of animals used would be reduced. This approach is strongly recommended when the animals involved are dogs or primates.

11.3.2.4. Data Sharing and Data Availability

The sharing of experimental data, whether it is positive or negative, can have an impact on reducing the unnecessary duplication of experiments, thus avoiding the unnecessary use of animals. This was commonplace in the US cosmetics industry as long ago as the 1970s, but has only recently begun to occur in the pharmaceutical industry. The promotion of data sharing is now an integral part of the new EU regulations for chemicals.

It is also important that data are made available for consideration by other scientists, so that improvements in experimental design, the optimal use of the information produced, and reductions in animal use can be achieved. Having data readily available also facilitates meta-analysis and the systematic review of data. Phillips notes that:

Meta-analysis of the existing literature allows conclusions to be made from a set of experiments that are more robust than subjective literature reviews, and should therefore, particularly in the case of conflicting results, reduce the number of animals required to undergo similar experiments. [17]

Systematic reviews are commonplace in the field of evidence-based medicine. Unfortunately, they are not yet widely used in relation to animal experimentation. This is partly due to the varied nature of animal studies, as well as to differences in the details provided when findings are published. Hooijmans *et al.* argued that journals should adopt a gold standard publication checklist, to ensure that the information in scientific papers is consistent and sufficiently detailed to permit systematic review and meta-analysis. [18].

11.3.2.5. Conclusion

What had been a downward trend in the overall numbers of animals used has been reversed in the last few years by the ever-increasing use of genetically-modified animals. Nevertheless, much more could be achieved, to the benefit of the biomedical sciences, as well as in terms of animal welfare, if a number of credible and well-documented opportunities for reduction were taken more seriously and were pursued more vigorously.

11.3.3. Refinement

Russell and Burch saw *refinement* as the third-ranking R, in that, if *replacement* is not possible and all attempts have been made to *reduce* the number of animals needed, then *refinement* comes into play. They felt that its purpose was:

To reduce to an absolute minimum, the amount of distress imposed on those animals that are still used. [1]

They regarded refinement as the most problematic of the Rs, given the diverse nature of experimental techniques applied to animals, yet, in more-recent times, it is the R where the most progress has been made. There is still much to be achieved, but the progress made has

partly been related to the incorporation of developments in animal ethics and animal welfare science. Buchanan-Smith *et al.* highlighted the historical definitions of *refinement*, and proposed the following harmonised definition:

Any approach which avoids or minimises the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved, and which enhances their well being. [19]

Refinement can be implemented in variety of ways, many of which are interrelated or overlap. Implementation can be restricted by the space available and by experimental protocols, and is more effective for some species than for others. It is also important to bear in mind that the behavioural and environmental needs of the animals can never be fully satisfied in a laboratory context. Decreasing the amount of distress animals encounter during their laboratory lives and improving their health status is key to ensuring high quality scientific output, as it reduces variance. [1, 20, 21].

11.3.3.1. Housing

The refinement of housing encompasses environmental enrichment to make the cages or enclosures more suited to the needs of the animals, as well as the grouping of animals together instead of keeping them singly. Animals can suffer, not only from the procedures they undergo, but also from being restricted socially, physically, and behaviourally. The improvements made can be as simple as providing nesting material for mice, or can involve more-complex structural changes, such as the introduction of ropes and swings in primate housing.

The provision of enrichment is controversial, since its advocates argue that enhancing animal welfare improves scientific validity, while its opponents feel that it introduces additional variables. At present, the evidence remains equivocal.

It is also important to understand the individual needs of different species, taking into account logistical restrictions, when developing and implementing enrichments or introducing group housing. Care must also be taken to ensure that there are no negative impacts on welfare, such as an increase in dominant behaviour, so that some animals in the group suffer more than when they were caged singly. Rennie and Buchanan-Smith discussed these nuances in the context of primates, but many of their points are also applicable to other species. [22].

11.3.3.2. Husbandry

Ensuring that traditional husbandry practices do not distress laboratory animals is also important to the improvement of their overall well-being. This is often linked to housing by ensuring that the environmental conditions, such as levels of temperature, humidity, light and noise, are appropriate, and not unduly disturbed by husbandry practices such as cage cleaning. While these basic conditions are essential, husbandry can be refined by ensuring that animals are handled correctly, or by implementing positive reinforcement training, so that animals (and in particular, the larger ones, such as dogs and primates) learn via a reward system to cooperate with everyday procedures, such as being dosed or being weighed.

11.3.3.3. Procedures

Refinement of the procedures applied to animals involves a vast number of issues and complications. For the purposes of generalisation in this chapter, we will refer only to changes to procedures that mean that they induce less pain, are less stressful or are then not required at all. This can be achieved, for example, by introducing pain relief, using non-invasive monitoring methods, developing a different procedure altogether, or acclimatising or training the animals. Various improvements in the use of rodents in anti-cancer drug development have both increased the relevance to the human situation of the data provided and enhanced the welfare of the animals used. [23] For example, highly-sensitive, bioluminescent imaging can be used to monitor very small cancers, which is a great improvement on the manual calliper measurements used in the past. As a result, the early stages of tumour development and the effectiveness of drugs can be studied, long before a tumour is large enough to have adversely affected the welfare of the animal. [24].

11.3.3.4. Humane Endpoints

Hau has provided a useful explanation of what is meant by ‘humane endpoint’:

The term humane endpoint indicates that certain endpoints in biomedical research are not humane, and that it is possible to introduce more humane endpoints in certain experiments by terminating them at an earlier stage, such as when clinical or physiological changes indicative of the effect of a treatment or procedure become identifiable. [25]

The imaging of tumour cells, as described above, is an example of the application of a humane endpoint, in that the animals do not have to suffer the debilitating effects of bearing a large tumour.

In 1998, a conference on humane endpoints concluded that they should be evaluated on a study-by-study basis, and that their development required input from several parties, including scientists, animal welfare officers and animal care staff. They should be reviewed regularly and should evolve as scientific developments take place. [26] An important factor in implementing humane endpoints is the development and use of appropriate methods for monitoring the health and well-being of the animals involved. Monitoring should be performed on a regular basis, by trained personnel.

11.3.3.5. Experimental Design

As well as being a tool for achieving reduction, good experimental design is also a means of achieving refinement in the same experiment. When planning an investigation, the whole programme of work should be considered, not just the individual procedures it involves. Careful consideration of objectives and the sequence of the procedures can lead to a significant refinement of the severity of the methods involved. Fry *et al.* provided examples of how this can work in practice. [27] This should involve ensuring that the proposed procedures and sampling methods are reviewed, and that refinements and/or less severe alternatives are considered. It may involve a consideration of whether the objectives of the programme could be achieved, if the lowest severity procedures were conducted first, and procedures involving higher severity were only justified by the success of the initial, low-severity studies. The use of pilot studies, and of different types of designs, such as factorial

designs, will also contribute to the minimising of severity or to reducing the number of animals that have to undergo more-severe procedures.

11.3.3.6. Conclusion

The aim of this section was to give the reader an overview of what is meant by *refinement* and how it might be implemented. We should add a caution that *refinement* and *reduction*, whilst often working together, can sometimes come into conflict, as on occasions where a decision has to be taken as to whether it is preferable to subject more animals to a less-severe procedure than to subject fewer animals to a more-severe one. *Refinement* should be the easiest R to implement, but much still needs to be done to increase awareness and implementation, including increased education and training, and greater dissemination of the resultant good practice. [28].

11.3.4. Experimental Design and Analysis

As was noted earlier, *reduction* is an important means of decreasing the number of animals that have to experience costs to gain the benefits of advances in biomedical science. Perhaps the easiest and most effective way to achieve reduction is through efficient experimental design and the appropriate use of statistics.

As was stated above, knowledge of these factors tends to be lacking among biomedical scientists. [29] While an in-depth description of these principles is not possible here, Festing *et al.* [30] and Ruxton and Colegrave [31] have provided greater detail in their texts on the subject.

11.3.4.1. Strategic Planning

The principles of reduction should be invoked, well before any practical work is conceived. By this, we mean that scientists should think through the entire process of the scientific investigation, from the initial definition of its aims and objectives, through the performance of the experiments, to how the findings would be analysed and applied. Evoking the terminology used by Russell and Burch in describing this as ...*the right choice of strategies in planning and performance of whole lines of research*, [1] members of the FRAME Reduction Steering Committee have developed a strategic planning scheme (Figure 11-1), which details the steps and thought process that scientists should go through when contemplating and conducting any research programme, but in particular, for those where animals might be involved. A detailed discussion of each of the steps has been provided by Gaines Das *et al.* [32].

11.3.4.2. Efficient Experimental Design

As well as contemplating the investigation as a whole, scientists should think carefully about how each individual experiment should be conducted, in order to gain the maximum scientific output from the minimum number of animals or, indeed, from non-animal resources or procedures.

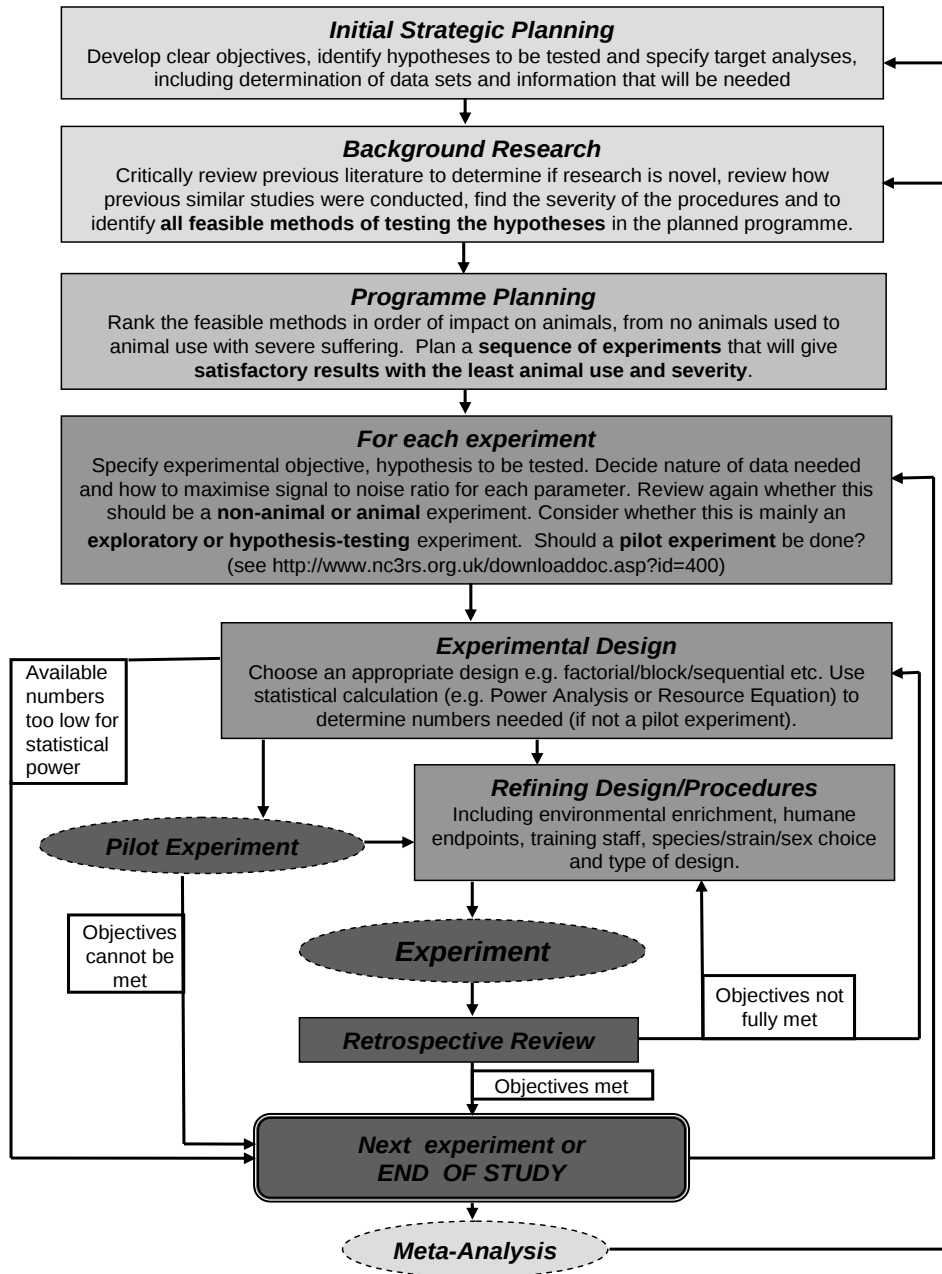


Figure 11.1. The FRAME Reduction Steering Committee Strategic Planning Flowchart, lightly modified and reproduced with permission from Gaines Das *et al.* [32].

Correctly designing an experiment can dramatically reduce the number of animals and other resources used, thus significantly lowering costs, both financially and in terms of personnel time. If the experiment was too big and more data were collected than was necessary for meeting its aims, then time, money and effort will have been wasted, and an unnecessarily large number of animals may have been subjected to unjustifiable suffering. Conversely, if the experiment was too small, then there may have been no chance of detecting

an effect, and the study may have had to be repeated — rather than saving resources and animals, more may be used in the end than would have been necessary, had the experiment been correctly designed at the outset. There are various types of designs, of a range of complexities, that can be used to improve the efficiency and quality of the scientific output, such as block, factorial, repeated measure and sequential designs, all of which are well-covered in the Festing *et al.* [30] and the Ruxton and Colegrave [31] texts.

11.3.4.3. *Appropriate Statistics*

As with an optimised experimental design, the statistical procedures to be used should be predetermined, to ensure that relevant and testable data are collected from the experiment. Statisticians are not magicians, and they cannot be expected to work miracles by identifying something useful at the end of a poorly-designed project, just because they are presented with reams of data. It is important to remember that it is the quality of the data that counts. Even small quantities of data can be analysed with powerful statistical methods, if they have been collected in such a way that they meet the assumptions of those statistical tests. If no way to analyse the data can be found, or if the data do not enable the aims of the study to be met, then time, materials, money and animal lives will have been wasted. The best approach is to consult a statistician in the initial planning phase of the investigation, rather than at the end, in the hope that something can be rescued. That is the way to ensure that the experimental design used is sufficient to provide data that will meet the assumptions of the proposed statistical test, and will provide valid answers to the scientific questions being asked, while making the conclusions more robust.

11.3.5. Replacement

Russell and Burch defined a *replacement technique* as:

Any scientific method employing non-sentient material, which may in the history of experimentation replace methods which use conscious living vertebrates. [1]

They distinguished between *absolute replacement*, where sentient vertebrates are not required at any stage, and *relative replacement*, which includes non-recovery experiments on intact, living, but completely anaesthetised animals, and the painless killing of animals to provide cells and tissues. Nowadays, attention tends to be focused on *replacement alternative methods*, which include the use of lower organisms, the early embryonic stages of vertebrates, isolated *in vitro* sub-cellular, cell and tissue preparations, and cell, tissue and organ cultures, computer based (*in silico*) models, and even the ethical use of human volunteers. It is also useful to distinguish between *direct replacement*, in which a method provides more or less the same information as would have been provided by an animal experiment, and *indirect replacement*, in which the information provided is different in kind, but can be employed for a purpose similar to that for which animal data would have been used.

Another important distinction is between the use of animals and replacement alternative methods in basic research and in efficacy and safety/toxicity testing. The development of methodology in basic research is continuous and relatively informal, being subjected to peer review in various ways (e.g. reviews of funding applications and of manuscripts submitted for

publication). In the case of the applied sciences, including chemistry, pharmacology and toxicology, there are formal and staged processes between development and acceptance into use, since they involve methods which are subject to approval and application, in line with unavoidable legal and regulatory requirements laid down by governments and intended to protect people and the environment.

It is for these reasons, and because the tests concerned often inevitably involve what Russell and Burch saw as direct inhumanity, that targeted efforts to find replacement alternatives have tended to be focused on the applied sciences, fully taking into account the fact that, whatever the biological system under consideration, there are two kinds of important outcome when a bioactive compound interacts with it: pharmacodynamic (PD) or toxicodynamic (TD) events (what the compound does to the system), and pharmacokinetic (PK) or toxicokinetic events (what the system does to the compound).

The enormous amount of effort invested in biological and computational technology in recent years has resulted in the availability of a vast and ever increasing array of tools for use as replacement alternatives. Many of these tools are based on human material or human experience, so the limitations of non-human models and the problem of extrapolation of information derived from them can be avoided.

The use of *computers* will be essential in virtually every aspect of the further development and use of the new technologies, as a means of collecting, storing, organising and analysing data, and detecting associations and correlations which deserve further attention. Therefore, while recognising the interdependence of most, if not all, the new technologies, we will use *in silico* as a term to distinguish certain computer-based approaches from approaches described by older terms, e.g. *in vivo*, *ex vivo* and *in vivo*. In the same way, while *informatics* applies broadly to the application of all the other technologies, it has a more-precise use in *bioinformatics*.

Descriptions of the available and developing technologies which can contribute to the replacement of animal procedures, could be based on various classifications and subdivisions (Table 11-1). There is a vast and rapidly-expanding literature on this subject, and all we can do here is to give a few examples, including some recent and comprehensive reviews. [33-35] The following summaries are intended principally to indicate the variety of these tools and to briefly consider only some aspects of their use.

1. *The use of existing knowledge.* The consideration of any novel compound or preparation should always begin with a search of the literature and the consultation of in-house data banks and other, more widely-available, resources.

2. *In chemico analysis and chemical interactions.* The physicochemical properties of molecules are significant and measurable, including stability under various conditions, volatility and acidity/alkalinity. For example, compounds with a low or high pH can cause severe and direct damage to the eye or the skin because of this, irrespective of the properties of the eye or skin themselves, so it is not necessary to test them with living material, *in vivo* or *in vitro*. The covalent binding of xenobiotic molecules, including drugs, to biological macromolecules, such as proteins, can affect their uptake into the body, their passage across membranes, and their half-lives in the blood or body tissues.

3. *In silico methods.* There are many computer-based analytical approaches, from (quantitative) structure–activity relationships ([Q]SARs) to category formation and read-across, which are combined with database compilation and the integration of information from different sources. The use of *in silico* methods in drug discovery was discussed in detail

by Ekins *et al.*, who stressed the importance of integrating computational and experimental data. [36, 37] *In silico* methods are increasingly used in toxicology, [38] A comprehensive consideration of principles and applications in *in silico* toxicology has recently been published, which deals, *inter alia*, with the development of QSAR and other models, and the procedures for assessing their quality and applicability for the prediction of toxic hazard. [39]

4. *In vivo studies on lower organisms.* Many types of organism undoubtedly have value for studies at the basic research level, including plants such as onion (*Allium cepa*) and garlic (*Allium sativum*), bacteria such as *Escherichia coli*, fungi such as yeasts (*Saccharomyces cerevisiae*), coelenterates such as hydra (*Hydra magnipapillata*), nematode roundworms such as *Caenorhabditis elegans*, insects such as *Drosophila melanogaster*, lower vertebrates, including fish such as the zebrafish (*Danio rerio*) and amphibians such as the South African clawed toad (*Xenopus laevis*). These organisms are being extensively used in research on cell and molecular biology, cell death, ageing, developmental biology, immunobiology and neurobiology. However, although some pharmacotoxicological test systems involving lower organisms and aimed at predicting effects in humans have been proposed, they are unlikely to provide satisfactory solutions, because the differences between these organisms and humans are too great for tackling other than certain highly specific questions (e.g. what is involved in DNA damage and repair).

5. *In vitro systems.* A wide variety of *in vitro* systems are available, which range in complexity from the use of isolated cell fractions over a few hours to the long-term maintenance of cells and tissues in multi-organ bioreactors (Table 11-2). By definition, “culture” is applied to cell, tissue or organ preparations which can be maintained *in vitro* in a nutrient medium for more than 24 hours. The trend is toward greater sophistication and greater humanisation, which leads to greater physiological and pharmacotoxicological relevance, but also to higher and higher costs in terms of time and human and economic resources. [40-44].

Table 11.1. Systems and approaches which can contribute to the replacement of animal experimentation

1. Use of existing knowledge
2. <i>In chemico</i> analysis and chemical interactions
3. <i>In silico</i> methods
4. <i>In vivo</i> studies on lower organisms
5. <i>In vitro</i> methods (Table 11-2)
6. High-throughput screening
7. High-content screening
8. Omics approaches
9. PBK models
10. Virtual tissue models
11. Human volunteer and clinical studies
12. Virtual patient populations
13. Biomarkers
14. Clinical imaging
15. Informatics
16. Systems biology
17. Integrated testing strategies

Table 11.2. *In vitro* systems and approaches which can contribute to the replacement of animal experimentation

Cell fractions
Primary cell monolayer or suspension cultures
Continuous cells lines
Immortalised cell lines
Stem cells
Genetically-engineered cells
Co-cultures
Organotypic cultures
Precision-cut slices
Perfused cultures
Reconstituted tissue equivalents
Engineered tissues
Dynamic bioreactors
Multi-organ systems
Cell-/organ-/human-on-a-chip

6. *High-throughput screening*. Made possible by advances in robotics and computer technology, high-throughput screening (HTS) involves the rapid testing of huge numbers of compounds for selected activities or interactions with specific proteins, receptors or other cell components. Methods including drop-based microfluidics can now permit 100 million reactions to be conducted in 10 hours. [45] In drug discovery, selection on the basis of HTS can be followed by lead optimisation, which can involve the synthesis of new analogues with improved potency, reduced off-target activity, and properties indicative of manageable *in vivo* pharmacokinetics, as well as *in silico* analyses.

7. *High-content screening*. Originally developed as a drug discovery method, high-content screening (HCS) permits the evaluation of multiple biochemical and morphological parameters in cells. [46, 47] For example, fluorescent tags or fluorescent antibodies can be used to detect proteins of interest via the parallel use of spatially or temporal resolved methods to provide multiple sources of quantitative information suitable for integrated analyses. Changes in cells in response to potential drugs or toxicants can be detected with high resolution in automated systems. HCS can be used, for example, to look at test item–cell surface interactions, signal transduction cascades, and effects on the cytoskeleton, and can be linked through phenotypic/visual screening to various omics and other approaches relevant to target identification and responses, and to investigating the significance of genetic polymorphism. Although slower than high-throughput screening, HCS offers much more information.

8. *Omics approaches*. The sequencing of the human genome, and the development of a wide range of methods for application in molecular and cellular genetics, have opened up dramatic new possibilities for increasing our understanding of human diseases and devising effective and relatively safe ways of curing or managing them. [48] Hence, *pharmacogenetics*, a rather descriptive approach to differences between individuals in terms of disease and their responses to drugs, has been supplanted by *pharmacogenomics*, a dynamic approach to obtaining and using genetic information at the population level as a

basis for drug design and development, then as a basis for the management of drug therapy by choice of drug and selection of dosing regimen, followed by monitoring of positive and adverse effects in the individual patient.

The omics approaches themselves afford a wide range of tools with special uses, but which must be used in integrated and intelligent strategies. There seem to be an ever-expanding number of omics, including:

- *cellomics* (about phenotype and functions at the cellular level);
- *cytomics* (distinguishable from cellomics by its application at the single cell level);
- *epigenomics* (about changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence);
- *interactomics* (about interactions and their consequences among proteins and other molecules within a cell);
- *metabolomics* (about the chemical processes involving metabolites), which is related to:
- *metabonomics* (about quantitative, dynamic and multiparametric metabolic responses);
- *pharmacogenomics* (a generic term, as referred to above);
- *phenomics* (about the functional biochemical and physiological characterisation of cells, tissues and organisms in response to genetic changes and environmental influences);
- *proteomics* (about the proteome, the entire complement of proteins, and about their individual production, modification and functions);
- *toxicogenomics* (about responses to toxic substances);
- *transcriptomics* (about all the types of RNA, including mRNA, rRNA and tRNA, as applied to the total set of transcripts or a specific subset).

9. *Physiologically-based pharmacokinetic models.* *In vitro* tests, *in silico* modelling and the omics-based systems tend to concentrate primarily on potential pharmacodynamic and toxicodynamic events, but, if drugs are to be developed and chemicals and chemical products are to be used, and patients, workers and consumers are to benefit and to be protected, the information provided by these approaches is of little value, unless major biokinetic processes, and, in particular, absorption, distribution, metabolism and elimination (ADME), are fully taken into account. [49].

These questions are now approached through the use of biokinetic models, especially physiologically-based pharmacokinetic (PBPK) models, a term which will be used to cover toxicokinetic models as well. [50] PBPK model development takes account of ADME on the basis of interrelationships among the critical determinants of these processes, such as tissue volumes, flow rates, rates of absorption, diffusion across membranes, tissue: blood partition, and rates and affinities for biochemical reactions. PBPK models are designed to provide a representation of the intact organism, including routes of entry or uptake (via gastrointestinal tract, skin, lungs or blood), distribution (via bloodstream), target organs (e.g. liver, kidneys, brain), and storage sites (e.g. adipose tissue). Account can be taken of the differences between rapidly-perfused tissues (liver, kidneys, brain) and slowly-perfused tissues (muscle, skin). Equations can be derived for describing features such as tissue influx and efflux, hepatic

metabolism, and renal clearance. The data on which the model is developed are obtained from the literature, from physicochemical considerations, from *in silico* approaches and *in vitro* tests, and from experiments on animals and, where ethically acceptable, studies on humans or human preparations and tissues obtained from organ donors. PBPK models can be used to guide dose selection, as well as to indicate responses to be looked for in later stages of the drug development process.

10. *Virtual tissue modelling.* The first mathematical model of the working heart was produced by Denis Noble in 1960. [51] A number of other models are now available, in which the virtual tissues are biophysically and anatomically detailed, and provide quantitatively predictive models of the physiological and pathophysiological behaviour of the tissues within the isolated organ. For example, a model of the heart involves the reconstruction of the electrical activities of cardiac tissues by producing computer models in the form of virtual tissues, and a model of uterine tissues can be used to identify possible mechanisms involved in premature labour. [52].

11. *Human volunteer and clinical studies.* The ethical and strictly-controlled use of human volunteers and patients can provide samples of body fluids from individuals with and without particular diseases, as well as subjects to be involved in clinical trials. The samples are essential for the application of the omics approaches and *in vitro* systems, but problems with the safe and ethical provision of cells and tissues for use in the *in vitro* approaches summarised in Table 1 can be limiting factors for progress with this type of replacement alternative.

12. *Virtual patient populations.* This novel approach can be used to conduct virtual clinical trials for efficiently screening drug candidates, and for evaluating the prospect that they could be brought to the market successfully. [53].

13. *Biomarkers.* As used in pharmacotoxicology, biomarkers are objectively measured and evaluated indicators of normal biological processes, pathogenic processes, and (desired) responses to or (adverse) effects of deliberate, incidental or accidental exposures to chemicals, drugs and other chemical products or to pathogens and biological products, such as vaccines. It is useful to distinguish between disease-related biomarkers and drug-related biomarkers, and they can also be subdivided into various categories, such as biomarkers of susceptibility, of exposure, of drug efficacy, of toxicity, or of patient response, or as diagnostic biomarkers. Another distinction is between imaging biomarkers and molecular biomarkers. They should be specific to particular circumstances and processes, reliably measurable, and fit for use for a defined purpose.

Biomarkers can provide vital and exploitable links between all the elements in the new pharmacotoxicological technologies, from computer-aided drug design, via *in silico* modelling for efficacy and toxicity, through the use of the omics, medium-throughput and high-throughput screening and HCS, along with the use of *in vitro* systems at various levels of sophistication, to PBPK modelling, and eventually to the investigation of polymorphism within the human population, as a basis for planning and monitoring therapies designed for the individual patient or protecting workers and consumers from damage caused by exposure to harmful chemicals and products.

The principal way of discovering biomarkers is by the application of the omics approaches in the toolbox, linked with bioinformatics, but most importantly, also with clinical observations and analyses. Their development involves five main stages: target biomarker identification (relevant to a given disease or drug therapy); clinical characterisation (in patient

with/without the disease or using/not using the drug); retrospective repository studies (on samples relevant to the disease or the use of the drug); prospective screening studies (to predict the occurrence of the disease or the effects of using the drug); and clinical use (in control of the disease or management of the use of the drug). For example, Vasan [54] and Corrias *et al.* [55] have discussed biomarkers in relation to cardiovascular disease and cardiotoxicity, and the US Food and Drug Administration (FDA) has formally accepted seven renal safety biomarkers for use in the non-clinical and clinical stages of drug development. [56]

14. *Clinical imaging.* The application of clinical imaging techniques to patients is likely to be of increasing value, as they are non-invasive and can produce multi-dimensional results, which can be both qualitative and quantitative, which can be used in association with information obtained by applying other technologies. [57] X-ray computed tomography (CT) has great promise, since, for example, it can produce a 3-D image of the heart and its blood supply from a series of 2-D images. Magnetic resonance imaging (MRI) can also be used to visualise internal structures, and is especially useful in showing what is happening in the brain tissues. Optical coherence tomography (OCT), now typically employing near infrared spectroscopy, can provide detailed images from within the retina; this can be used to assess axonal integrity in multiple sclerosis, and to visualise lipid-rich plaques in coronary arteries. Positron emission tomography (PET) is another way of producing images of what is taking place within the body. Used with the glucose analogue, fludeoxyglucose, PET can be used to measure which cells in the body take up glucose, and this can throw light on sites of inflammation and early tumour development. Thus, by applying clinical imaging techniques, the nature and progression of events can be followed at the molecular level, within the human body. In drug development, it is possible to develop PET imaging strategies to visualise drug interactions with the targets on cells.

15. *Informatics.* In its broadest sense, *informatics* encompasses information science and information technology (IT), is essential to the successful management of human activities of virtually all kinds, given the enormous amounts of information now available on all kinds of topics, not least through the Internet. At least three kinds of informatics technologies are involved in drug discovery, development and use: *cheminformatics*, the application of computer and IT techniques to problems in the field of chemistry, *health informatics*, where computer science and IT meet health care, and which includes *biomedical informatics* and *clinical informatics*; and *bioinformatics*, the application of computer science, IT and statistics in molecular biology, especially in the management and analysis of data provided by the omics approaches.

Bioinformatics involves the use of computer-intensive techniques to search through vast amounts of data, on, for example, DNA sequences and protein sequences and structures, in order to increase understanding and facilitate problem solving, by identifying patterns and correlations, and creating algorithms (mathematical formulae consistent with expressions of finite lists of well-defined instructions as a basis for guiding data processing and automated reasoning). This can provide, for example, a basis for the comparative analysis of genome content, of gene expression, of gene mutation, of gene regulation, of protein expression, of network modelling, and of molecular design, in ways which facilitate the identification of applicability domains and prediction in models in *in silico* modelling and *in vitro* testing [58].

16. *Systems biology.* Discussions on drug development frequently mention a systems biology approach, i.e. a multidisciplinary approach to considering the interactions between

the components of a biological system and combining this knowledge to increase understanding of the organism or of the phenomenon being considered. [59] In a way, this represents a holistic approach to combining the data provided by a variety of confirmatory or complementary reductionist approaches. Relatives of systems biology are *meta-analysis*, which combines parts of the results of several studies, and *evidence-based medicine* and *evidence-based toxicology*. [60].

17. *Integrated Testing Strategies*. Those with the responsibility for considering the potential benefits and costs of human exposure to chemicals and chemical products are now faced with an increasing complexity and variety of methods based on mechanisms of action and modes of action at the molecular, cellular and tissue/organ/system/organism levels. These methods are suited to answering highly specific questions, rather than to providing information on biological or toxicological effects of all kinds, as in the one-model-suits-all systems once thought to be provided by laboratory rats, mice, dogs or primates. This means that the available tests will have to be used intelligently and selectively in combination, as batteries and/or in tiered hierarchical, often decision-tree, schemes, in what have come to be known as Integrated Testing Strategies (ITS). [61, 62].

Before an ITS can be considered acceptable for use, especially in compliance with regulatory requirements, it must be established that it is relevant and reliable for a particular purpose (e.g. the classification and labelling of chemicals in terms of their toxicity, or for identifying particular hazards such as carcinogenicity, eye irritation, hepatotoxicity, nephrotoxicity, neurotoxicity, reproductive toxicity, skin irritation or skin sensitisation). [62] This, in turn, requires that principles for the development, evaluation, acceptance and use of ITS need to be established and agreed by all the stakeholders.

11.4. Legislation Governing Animal Experimentation

The laws governing the use of animals in experiments vary massively across the world, from virtually no or little control to extensive limitations and guidelines. Here, we will give an overview of the regulations applied by two of the largest users of laboratory animals, Europe and the USA, including a comparison of where they differ significantly. First, we will consider how the history of animal experimentation legislation developed in the UK. For information on the equivalent laws in other countries, see Cooper [63] and Radford. [64].

11.4.1. The Cruelty to Animals Act 1876, UK

The first legislation anywhere in the world to specifically lay down restrictions on the use of animals for experiments was the *Cruelty to Animals Act 1876* in the UK. [65] The Act came out of a report by the Royal Commission on the Practice of Subjecting Live Animals to Experiments, which was set up as result of mounting pressure from Victorian society, including members of the scientific community and groups such as the RSPCA and the National Antivivisection Society (then known as the Victoria Street Society for the Protection

of Animals from Vivisection), who were disturbed about animal experiments being conducted in Britain and in other countries. [65, 66].

The 1876 Act made it an offence to perform on a living animal any experiment calculated to cause pain, unless it was carried out with a view to advancing physiological knowledge, saving or prolonging life, or alleviating suffering. This led to the creation of a regulatory system involving the registration of premises where experiments were conducted and the licensing of people who conducted the work, enforced by a newly-established Government (Home Office) Inspectorate.

11.4.2. The Animals (Scientific Procedures) Act 1986, UK

A century after the 1876 Act came into force, pressure on the UK Government again built up, which included the promotion of alternatives to the use of animals in education, research and testing, and eventually resulted in the passage of the *Animals (Scientific Procedures) Act 1986* (ASPA). [67] The political processes leading to the eventual establishment of the Act were drawn out and complex, and are well-described elsewhere. [66, 68] However, of interest is the role that animal welfare and alternatives advocates had in the deliberations. The British Veterinary Association (BVA), the Committee for Reform of Animal Experimentation (CRAE) and the Fund for the Replacement of Animals in Medical Experiments (FRAME) formed an alliance, which advised the Government at all stages during the drafting of the eventual Act and its passage through Parliament.

The ASPA represented a substantial change to the regulatory procedures for controlling and overseeing the use of animals in scientific research. Unlike the 1876 Act, it encompasses many more procedures, animals and users of animals, including breeding establishments, and is accompanied by a detailed guidance document, the *Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986* (HOG), to show how the ASPA is to be applied in practice. [69] The underlying provision of the ASPA (Section 3) is that:

No person shall apply a regulated procedure to an animal unless:

- a) he holds a personal licence qualifying him to apply a regulated procedure of that description to an animal of that description;
- b) the procedure is applied as part of a programme of work specified in a project licence authorising the application, as part of that programme, of a regulated procedure of that description to an animal of that description; and
- c) the place where the procedure is carried out is a place specified in the personal licence and the project licence.

Under the ASPA, the numbers of animals used in scientific procedures and the procedures applied to them have to be reported annually, and the statistics are published by the Home Office. The Home Secretary is ultimately responsible for the enforcement of the ASPA, via the Home Office, with a dedicated Inspectorate which advises on the granting of licences, inspects establishments, and reports any breaches of licence conditions or certificates. The inspectors themselves do not actually have the power to grant, refuse, vary or revoke licences. However, when a protected animal is found to be undergoing excessive

suffering, the inspector can require that the animal be killed immediately by a humane method. Also related to the functioning of the ASPA are the Animal Procedures Committee (APC) and the Ethical Review Process (ERP). The APC is an independent statutory advisory body, established to provide strategic advice to the Secretary of State on policy, practice, ethics, science and welfare. The ERP is required by the ASPA, and should take place in each licensed establishment with the remit to:

- a) provide independent ethical advice to the certificate holder, particularly with respect to project licence applications and standards of animal care and welfare;
- b) provide support to named people and advice to licensees regarding animal welfare and ethical issues arising from their work; and
- c) promote the use of ethical analysis to increase awareness of animal welfare issues and develop initiatives leading to the widest possible application of the Three Rs.

While the ASPA does not explicitly mention the Three Rs, it does strongly imply their active implementation, for example, by stating in Section 5.5 that:

The Secretary of State shall not grant a project licence unless he is satisfied:

- a) that the purpose of the programme to be specified in the licence cannot be achieved satisfactorily by any other reasonably practicable method not entailing the use of protected animals; and
- b) that the regulated procedures to be used are those which use the minimum number of animals, involve animals with the lowest degree of neurophysiological sensitivity, cause the least pain, suffering, distress or lasting harm, and are most likely to produce satisfactory results.

The HOG goes further and puts this in the context of the Three Rs: in Section 2.2:

The Secretary of State must be satisfied that to achieve the purpose of the study there are no suitable alternatives that do not require the performance of regulated procedures on animals, and that full use will be made of reduction and refinement strategies to minimise the number of animals used and the likely pain, suffering, distress or lasting harm to be caused (Section 5[5]). Authorities cannot be granted when appropriate replacement, reduction and refinement alternatives are reasonably and practicably available.

The ASPA implements the requirements of the European legislation controlling animal experiments in *Directive 86/609/EEC*. [70] This Directive was revised in 2010, [71] and the UK Government is currently working toward transposing this into UK law. It is likely that a revision of the ASPA will be published in due course, but how substantial the changes made will be is not yet known.

11.4.3. European Directive 86/609 and Directive 2010/63, EU

Directive 2010/63/EU, [71] which has replaced Directive 86/609/EEC [70] on the protection of animals used for scientific and other experimental purposes, was adopted on 22

September 2010. The revision was deemed necessary to remove disparities in laws, regulations and administrative provisions among the Member States, taking into account the increase in the number of Member States since 1986. These disparities have resulted in vast differences in the levels of protection afforded to laboratory animals within the EU, which might lead to problems with trading products and substances that are developed through animal experiments. Each Member State is responsible for enacting the Directive by transposing it into its own legislation.

The new Directive establishes measures to protect animals used for scientific or educational purposes, by laying down rules on:

- a) the replacement and reduction of the use of animals in procedures and the refinement of the breeding, accommodation, care and use of animals in procedures;
- b) the origin, breeding, marking, care and accommodation and killing of animals;
- c) the operations of breeders, suppliers and users; and
- d) the evaluation and authorisation of projects involving the use of animals in procedures.

[71]

While this Directive could be seen as an improvement on its predecessor in terms of raising the minimum care standards and giving more-explicit recognition of the Three Rs, it still falls below the stricter conditions imposed by the national legislation of some Member States, such as the ASPA in the UK. Therefore, at the time of writing, there is some concern among advocates of both non-animal alternatives and animal welfare, as well as some other sectors of the scientific community, that this might lead to a weakening of some national legislation, when the aim was said to be to improve standards in those countries where requirements were less restrictive and/or where standards of animal care are lower.

11.4.4. Animal Welfare Act, USA

In the USA, what is now known as the *Animal Welfare Act* (AWA) sets the minimum standards of care and treatment for animals used in research. [72] It was first enacted in 1966 as *Public Law 89-544*, but was amended in 1970, as *Public Law 91-579*, when its scope was broadened to extend its coverage of animals used in experiments, and to include animals used in exhibitions and involved in the wholesale pet trade. Since then, it has been strengthened and broadened by further amendments. The US Department of Agriculture (USDA) enforces the AWA through the Animal Care Programme of the Animal and Plant Health Inspection Service (APHIS).

As with European Union Directive, the AWA lays down:

- minimum standards of veterinary care and animal husbandry;
- requirements for licensing of research facilities, but not of individuals;
- requirements for the provision of anaesthesia or pain relieving medication;
- requirements for facilities to provide dogs with the opportunity to exercise;
- requirements to promote the psychological well-being of primates;
- forbiddance of unnecessary duplication of a specific experiment; and

- requirements for the establishment of institutional animal care and use committees (IACUCs) to oversee the use of animals in experiments.

However, the US legislation is much less restrictive than that in force in Europe, and there are some important differences. The most startling of these is the exclusion of birds, rats, mice, fish, amphibians and reptiles from the definition of ‘animal’ in the AWA, so these animals are not protected. According to the Humane Society of the United States (HSUS), this means that approximately 95% of the animals used for research in the USA are not afforded even the minimal protections of the AWA. [73] A second difference is that the IACUC is the body responsible for ensuring compliance with the AWA and for providing documentation on all areas of that compliance to APHIS, so compliance is measured internally and not independently from the outside.

11.4.5. Conclusion

Even from its inception in the 19th century, the regulation of animal experiments has represented a compromise between the aims of science and the protection of animals and their welfare. This compromise is frequently seen as inadequate, by basic researchers wanting to pursue their scientific endeavours, and pharmacotoxicologists needing to comply with regulations, but no less by those who want to promote non-animal alternatives to animal procedures, and/or who desire to promote animal welfare and eliminate suffering caused to animals in the pursuit of science. The regulations governing animal experimentation vary greatly between countries and continents. This leads to problems with regard to animal welfare, animal breeding and supply, the scientific procedures used, the quality of the results obtained, and even to unfair trade practices. Greater harmonisation is an imperative need.

11.5. Testing Required by Regulations

Governments, industries, scientists, consumers, patients and workers are faced with the problem of evaluating the benefits of using potentially-hazardous chemicals and chemical products of many kinds in comparison with the risks which may result from exposure to them. This has resulted in a vast and complex array of laws, regulations, guidelines and practices, many of them resulting from action taken when something went wrong (e.g. the increase in reproductive toxicity testing as a result of the thalidomide tragedy). Many of the test procedures in place involve the use of laboratory animals. This is causing increasing concern, not just because of increased public awareness of animal welfare issues, but also because insurmountable differences between humans and laboratory animals can inhibit the acceptance and application of useful new chemicals, or limit the discovery, development and approval and acceptance of new treatments against disease, while at the same time leading to failure to detect adverse effects at the right time in product development and approval for marketing and use. One serious problem is that the regulations, and the ways in which tests are required and the data they produce are applied, are now so very complicated that only those directly involved, as manufacturers or regulators, can hope to understand them.

The evaluation of toxic hazard and the prediction of risk to humans must be focused on the following: a) the evidence that a given chemical or drug can cause adverse effect(s); b) the frequency of incidence of those effects in a given population; c) the degrees of severity of the effects; d) variations in susceptibility to and in the expression of the effects within the population and between populations; and e) the epigenetic factors which can modulate them. This is reflected in the regulations currently in place, which differ according to the types of item under consideration. We cannot consider all of them here, so we will confine our discussion to three important areas, namely, the regulation of chemicals, pharmaceuticals and medical devices.

11.5.1. The Regulation of Chemicals

Chemicals are regulated with regard to their manufacture, marketing and transport, and their use in thousands of different products, including cosmetics, household products, and pesticides. Their entry into the body is not usually intended, but can occur as a result of accidental, environmental or occupational exposure. There are a vast number of regulatory requirements, which are laid down and applied at national (e.g. Japan, USA), regional (e.g. EU) and international (e.g. UN) levels. It is important to recognise the differences between requirements concerned with when testing should be done, what tests should be done and how they should be conducted, and how the results obtained should be reported (e.g. in submissions to the appropriate authorities) and acted upon (e.g. via classification and labelling, restrictions on use and the issuing of warnings).

In the EU, chemicals regulation is now via the REACH (Registration, Evaluation, Authorisation, and Restriction of Chemical Substances) system, which is backed by EU legislation agreed in 2006 and is managed by the European Chemicals Agency (ECHA) in Helsinki, Finland. The REACH system came into force on 1 June 2007, and further information about its operation is available via the ECHA website (http://echa.europa.eu/home_en.asp). The ECHA operates in collaboration with the Competent Authorities in the Member States, e.g. the Health and Safety Executive in the UK (<http://www.hse.gov.uk/reach/>). In the USA, chemicals are regulated by the Environmental Protection Agency (EPA), according to the *Toxic Substances Control Act 1976* (<http://www.epa.gov/regulations/laws/tsca.html>), while in Japan, they are regulated by the Ministry of the Environment, according to the *Chemical Substances Control Law 1973*, as amended in 2009, as usefully summarised by Kazu Takemoto, the Vice-Minister for Global Environmental Affairs in 2010. [74] Over the last few years, many other countries have been in the process of strengthening their own requirements.

The regulations are focused principally on the prediction of hazard, and the methods used have traditionally been dominated by the use of high fidelity animal models. Testing is conducted according to international guidelines, primarily the OECD Health Effects Test Guidelines (TGs). Data produced according to these TGs (primarily for the manufacturers of chemicals) are then incorporated into the submissions made (primarily by the downstream users of the chemicals) to the national and international regulatory authorities responsible for the protection, not only of human health, but also for the protection of many other organisms and the environment in general, from the effects of exposure to hazardous chemicals. This should involve assessments of the likely routes, types and scales of human exposure, but all

too often, there is a routine, all-embracing check-list approach, as favoured by regulators. The complicated process involved in approving and updating the TGs is a matter of great concern in some quarters. [75].

Given the progressive globalisation of manufacturing and marketing, differences between regulations and how they are applied can lead to serious difficulties for companies and governments. A number of steps have been taken in attempts to resolve this problem. For example, the Globally Harmonised System of Classification and Labeling of Chemicals (GHS) is an internationally-agreed system to replace the various different classification and labelling standards used in different countries. [76] This is most encouraging, but serious differences remain, e.g. between the EU REACH and US TCSA systems. [77].

The implications of the original REACH proposals caused great alarm to both chemical manufacturers and their downstream user customers. In addition, animal welfare organisations and scientists spoke out against the proposed check-list approach to hazard prediction based on tonnage, which would fail to take sufficient account of the nature of the chemicals themselves or of likely human exposure. There have been many political and administrative developments in relation to the REACH system proposals since they were first put forward in 2001, and there is now a vast and burgeoning literature on the subject, including thousands of pages of guidance from the ECHA.

One major problem with the REACH system is the requirement to identify Substances of Very High Concern (SVHC), i.e. CMR chemicals (those which are carcinogenic, mutagenic or reprotoxic), or are PBT chemicals (those which are persistent, bioaccumulative and toxic), or are identified as SVHCs on a case-by-case basis from scientific evidence. Consultations on specific proposals are regularly announced on the ECHA website. However, attempts to identify potential human carcinogens are very unsatisfactory, since they rely on the rodent bioassay, which is fundamentally flawed, since it cannot be trusted to reliably identify *rodent* carcinogens, let alone *human* carcinogens. [78] It is estimated that at least 60% of the animal testing required by the REACH system will be for reproductive toxicology, and there is pressure from some traditional toxicologists and regulators to have this testing based on the two-generation test in rats, sometimes with additional testing in a second species, such as the rabbit. However, whether such an approach can be justified on scientific grounds is highly questionable, as it is unlikely that it would be successful in identifying the very small number of human teratogens likely to be found among the 10,000 or so “existing” chemicals which are of potential CMR concern. [79] The development of human-oriented strategies for identifying reproductive toxins and chemical carcinogens, involving *in vitro* and *in silico* procedures, should therefore be a matter of the highest priority.

These issues were discussed in detail by Hartung, who said that the difficulties introduced by the REACH system were so very great that they could lead to the more-active search for non-animal tests and testing strategies, and the application of evidence-based toxicology approaches. [80] We have long been calling for a revolution in toxicity testing based on new technologies. [61] That is also the theme of the 2007 report of the US National Academy of Sciences on behalf of the EPA, entitled *Toxicity Testing in the 21st Century: A Vision and a Strategy*, which spells out a much more intelligent approach than that currently being followed in Europe, emphasising the need to benefit from experience in the pharmaceutical industry, and to benefit from the emerging fields of systems biology and bioinformatics. [81].

11.5.2. The Regulation of Pharmaceuticals

Unlike most chemicals and chemical products, medicines are designed to be deliberately taken into the body, there to exert powerful effects on the body's cells, organs and systems, in order to assist in the diagnosis, treatment or prevention of disease. Given the complexity of the body and its control systems, it is not surprising that they can also induce adverse and serious side-effects. The principal aim in drug discovery and development is to identify compounds which will evoke the maximum desired therapeutic response according to dosing regimens which induce only minimal and manageable adverse effects.

The testing of pharmaceuticals differs from the testing of chemicals, since the exposed population is known, as are the dosing regimens applied. In addition, *in vivo* testing ultimately means testing in humans, during clinical trials and when an approved drug is used on a population scale.

In the EU, medicines can be licensed in two ways — via the national control agency (the Medicines and Healthcare products Regulatory Agency [MHRA] in the UK (<http://www.mhra.gov.uk/Howweregulate/index.htm>) or via the EU authority, the European Medicines Agency (EMA; formerly the European Medicines Evaluation Agency [EMEA]; http://www.ema.europa.eu/ema/index.jsp?curl=/pages/home/Home_Page.jsp), based in London, UK. Licensing via the EU is also accepted in some non-EU European countries. The equivalent authority in the USA is the Food and Drug Agency (FDA; <http://www.fda.gov/Drugs/default.htm>), and in Japan it is the Ministry of Health, Labour and Welfare (MHLW [82]).

On the whole, whereas definitive guidelines are laid down by the regulatory authorities for chemicals and many other chemical products, pharmaceutical companies tend to be able to discuss a promising new compound with the appropriate medicines control agency or agencies, before all the testing has been completed. In addition, since its inception in 1990, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH; <http://www.ich.org/home.html>) has been bringing together the regulatory authorities and pharmaceutical industries of Europe, Japan and the USA, to discuss scientific and technical aspects of drug registration. ICH is concerned with the design, conduct, safety and reporting of clinical trials, and has produced a comprehensive set of safety guidelines to uncover potential risks such as carcinogenicity, genotoxicity and reprotoxicity. [83] The process does not always work smoothly, as, for example, there appear to be regional differences on the value of using non-clinical data in assessing the risk of delayed ventricular repolarisation (QT interval prolongation), which has been one of the most important causes of drug withdrawal in recent years. [84].

The pharmaceutical industry is currently in a state of crisis, because of the increased costs of drug discovery and development, and the fact that, despite the increased numbers of candidate compounds in the development pipeline, the rate of entry to the market has steadily decreased, while the rate of post-marketing withdrawal, because of lack of efficacy or unpredicted adverse effects, has increased.

A variety of causes have contributed to this situation. The new targets to be tackled are more difficult than those tackled in the past, and there is insufficient understanding, not only of diseases themselves or of the mechanisms of the pharmacological and toxicological responses and effects involved, but also of the influence of human polymorphisms and the effects of a variety of contributory epigenetic factors. There has been a growing recognition

that differences among patients can affect not only the efficacy or safety of a drug, but even the results of clinical trials (since a sizeable cohort of non-responders or idiosyncratic responders can throw doubt on the efficacy of a drug which is effective for the majority of the individuals in the trial).

A further complicating factor is that the currently-available non-clinical tests and testing strategies, which are heavily dependent on laboratory animal tests, have failed to adequately predict adverse clinical effects, including the main causes of late drug withdrawal, namely, damage to the cardiovascular system, the liver and the respiratory system. [85] This is partly because the animal models used in various testing strategies are not sufficiently closely-related to what is being modelled, and therefore cannot be expected to provide a sufficiently relevant or reliable basis for making important decisions.

As a result of this, the need to fundamentally reappraise the value of animal studies as an essential and required background to human studies is increasingly being emphasised. The US Food and Drug Administration (FDA) put it like this in 2004, in *Challenge and Opportunity on the Critical Path to New Medicinal Products*:

Tools for safety assessments include product testing (e.g. for contamination), as well as in vitro and animal toxicology studies, and human exposure. Despite some efforts to develop better methods, most of the tools used for toxicology and human safety testing are decades old. Although traditional animal toxicology has a good track record for ensuring the safety of clinical trial volunteers, it is laborious, time-consuming, requires large quantities of product, and may fail to predict the specific safety problem that ultimately halts development. Clinical testing, even if extensive, often fails to detect important safety problems, either because they are uncommon or because the tested population was not representative of the eventual recipients. Conversely, some models create worrisome signals that may, in fact, not be predictive of a human safety problem. [86]

The FDA document goes on to say that:

There are currently significant needs, but also significant opportunities, for developing tools that can more reliably and more efficiently determine the safety of a new medical product. ... Proteomic and toxicogenomic approaches may ultimately provide sensitive and predictive safety assessment techniques; however, their application to safety assessment is in early stages and needs to be expanded. Targeted research aimed at specific toxicity problems should be undertaken. ... As biomedical knowledge increases and bioinformatics capability likewise grows, there is hope that greater predictive power may be obtained from in silico (computer modelling) analyses such as predictive toxicology. Some believe that extensive use of in silico technologies could reduce the overall cost of drug development by as much as 50%. ... FDA's files constitute the world's largest repository of in vitro and animal results that are linked with actual human outcomes data. Further data mining efforts that effectively protect proprietary data could form the basis for useful predictive safety models.

The demonstration of efficacy is also addressed by the FDA:

Predicting and subsequently demonstrating medical utility (also called benefit or effectiveness) are some of the most difficult and important challenges in product development. Currently available animal models, used for evaluating potential therapies prior to human clinical trials, have limited predictive value in many disease states. Better predictive

non-clinical screening methods are urgently needed. In many cases, developers must gamble on the results of the large-scale, expensive trials necessary to assess effectiveness in people. Such human trials are currently highly empirical, because most sources of variability in human responses are not understood and thus cannot be controlled for. It is clear to many in the field that new scientific advances have the potential to revolutionise clinical development.

This remarkable FDA initiative presents a great challenge and an enormous opportunity, which should be welcomed and responded to by all concerned, in the interests of good science and human benefit, as well as animal welfare. Those who have a tendency to want to protect the *status quo* at all costs, should note that phrases such as “*animal toxicology ... may fail to predict the specific safety problem that ultimately halts [drug] development*”, and “*currently available animal models ... have limited predictive value in many disease states*” have been used by the FDA, and not by animal rights protagonists alone.

These issues were brought sharply into focus by the very serious situation which arose in March 2006, when eight healthy male volunteers took part in a Phase I trial on an anti-inflammatory monoclonal antibody, TGN1412. [87] Six of the volunteers rapidly developed multi-organ failure, one of whom will suffer long-term disability. Preclinical studies had been conducted with a number of animal models, including monkeys, but no effects were seen which discouraged the manufacturer from proceeding to seek permission to conduct the Phase I trial, or the regulatory authority from granting its permission. The problem appears to have stemmed from the fact that the antibody was fully humanised, i.e. it was an antibody with human-specific properties, derived by protein engineering from an antibody produced in a non-human species. It could be said that the assumption that animal models could be used to establish the safety of such a product is a supreme example of the danger of the high fidelity fallacy. It seems likely that this tragic event will lead to fundamental changes in the way that preclinical and clinical studies on new medicines will be conducted in the future, especially as more and more “humanised” products are likely to be developed. [88].

Meanwhile, in Europe, the Innovative Medicines Initiative (IMI) has been established, as a joint undertaking between the European Union and the European Federation of Pharmaceutical Industries and Associations (EFPIA), with the aim of improving the drug development process by supporting more-efficient drug discovery leading to the development of better and safer medicines for patients (<http://www.imi.europa.eu/>). The IMI supports collaborative research projects and builds networks of industrial and academic experts in Europe. The focus is on the development and use of *in silico* and *in vitro* methods, and omics and imaging approaches, so that the new technologies can be used for the benefit of patients, as a result of dynamic interactions between what takes place in the laboratory and in the clinic.

11.5.3. The Regulation of Medical Devices

Unlike most industrial chemicals, medical devices are intended to be brought into contact with the human body, but they are not designed to effect the body's cells, tissues and systems in ways comparable with those for which medicines are designed and used. The definition of medical devices used by the International Organisation for Standardisation (ISO) [89] includes:

Any instrument, apparatus, implement, machine, appliance, implant, in vitro reagent or calibrator, software, material or other similar or related article, intended by the manufacturer to be used, alone or in combination, for human beings for one or more of the specific purpose(s) of:

- diagnosis, prevention, monitoring, treatment or alleviation of disease,
- diagnosis, monitoring, treatment, alleviation of or compensation for an injury,
- investigation, replacement, modification, or support of the anatomy or of a physiological process,
- supporting or sustaining life,
- control of conception,
- disinfection of medical devices, or
- providing information for medical purposes by means of in vitro examination of specimens derived from the human body,

and which does not achieve its primary intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means.

ISO is a worldwide federation of national standards bodies, which establishes technical committees to draft international standards on a wide range of topics. Technical Committee ISO/TC 194 produced *ISO 10993-1 on Biological evaluation of medical devices*, which, now in its fourth edition. [89] *ISO 10993-1:2006* is backed by 19 other parts, on evaluation and testing within a risk assessment process, on animal welfare requirements, and on 17 different kinds of test.

In June 2009, CEN, the European Committee for Standardisation published *EN ISO 10993-1:2009*, which, with its parts, is identical to *ISO 10993-1:2003*, except for some information annexes. [90] It is the European Standard, and the EU Member States publish their own versions to comply with it. In the UK, the British Standard is *BS EN ISO 10993-1:2009*, which is managed by the MHRA. [91] In the USA, regulation is the responsibility of the FDA, and the FDA Center for Devices and Radiological Health (CDRH) operates its CDRH Standards Program with the cooperation of the American Association for the Advancement of Medical Instrumentation (AAMI) and the American National Standards Institute (ANSI), and recognises the ISO standard via *AAMI/ANSI/ISO 10993-1: 2009*. [92] In Japan, earlier regulations were very stringent, with little room for interpretation, but the Japanese MHLW and its Pharmaceutical and Medical Device Agency (PMDA) were involved in the development of *ISO 10993-1*. Nevertheless, there can be technical specifications which are different from those of the ISO standard, and this could result in the need for additional testing. [93] Much has also been achieved via the Global Harmonisation Task Force (GHTF; <http://www.gh tf.org/>), but a comparison of the regulations of the EU, Japan and the USA and differences in the ways in which they are applied, suggests that there is still much to be done. [94]

ISO 10993-2:2006, on Animal welfare requirements, is an outstanding document. [95] It contains useful definitions of terms, including alternative method, animal, animal test, humane endpoints, protocol, reduction, refinement, replacement and validation. It emphasises the importance of establishing a framework that reflects the relevant ethical and legal

considerations, and of formal justification for using animal tests. It goes on to stress the importance of the competence of the personnel involved, of the proper planning and performance of animal tests, the strategic use of in vitro and in vivo tests in tiered or hierarchical approaches, ethical review, and the validation of test results and the mutual acceptance of data.

ISO 10993-2:2006 should be read and rigorously applied, not only by those concerned with medical devices, but by all those concerned in any way with the use of animals and non-animal methods in the testing of chemicals and many other products. Those who drafted ISO 10993-2:2006 deserve the gratitude and congratulations of all us. It is, of course, important to recognise that the many structural and physiological differences between laboratory animals and humans, which result in insurmountable hurdles to the use of animal test procedures to reliably predict effects and responses in humans, must apply no less to medical devices than to chemicals, pharmaceuticals and other products. This recognition is implicit in ISO 10993-2:2006, but how does it affect medical device testing in practice?

11.5.4. Conclusion

Driven by the concern that not enough is known about the safety of many chemicals in widespread use, that it is becoming increasingly difficult to succeed in getting drugs from discovery through preclinical and clinical testing and into use, and that traditional animal test procedures cannot be relied upon to provide the information that is needed, much effort is currently being invested in the elaboration, evaluation and implementation of testing strategies which take advantage of the many technological developments that are taking place. There are encouraging signs that novel approaches will be found for adoption, for example, in cardiotoxicology [96] and in inhalation toxicology, [97] and FDA, IMI and other initiatives are being intensely focused on drug-induced liver injury. [98, 99] Thought is also being given to how data obtained with non-animal test procedures can be used in novel risk assessment strategies. [100] Less encouraging is a lack of progress toward replacing animal tests for carcinogenicity and reproductive toxicity, and the European Commission is about to propose yet another postponement of the long-sought ban on the use of animal procedures for cosmetic ingredient testing. [101].

11.6. Testing Related to Dental Implants

Questions related to dental implants and their uses will be dealt with in great depth in the other chapters in this book. Nevertheless, we will endeavour to provide an overview of the regulations concerned with the safety testing of implants and their consequences, as viewed from our perspective and in the light of the information presented and opinions expressed earlier in this chapter.

In the EU, medical devices are classified according to guidance document *MEDDEV 2.4/1 Rev 9: 2010*, [102] and the associated classification of dental devices and accessories, *CEN/TR 1401: 2009*. [103] Dental implants are subject to Rule 8 (*for surgically invasive, long-term use, implantable devices*), and classification IIA (*to be placed in the teeth*). This

classification also applies to bridges and crowns, and dental filling materials and pins. Implants intended to be in place for more than 30 days are regarded as being “permanent”.

A dental implant can be defined as “a titanium root, used in dentistry to support restorations that resemble a tooth or a group of teeth to replace missing teeth”. Nowadays, the implanted roots can also be made of zirconia (zirconium dioxide). They are used as endosseous implants, i.e. they are placed within the bone, which osseointegrates the artificial root, so that it has the structural properties of a natural root, onto which a bridge or a crown can be added. There is disagreement in the dental profession about the time needed for osseointegration before a restorative bridge or crown is added to an implant. The condition of the bone which is to receive the implant is particularly important, and computer software is now available for virtual surgical implant placement, which takes account of bone quality, the need for bone grafting, implant characteristics and implant placement.

The insertion of a dental implant can fail because of inadequate osseointegration. Problems can also result from peri-implant inflammation, and in patients who have poor oral hygiene, are heavy smokers, or have type 2 diabetes, or in patients taking certain drugs (e.g. anticancer drugs).

Two main types of consideration affect the testing of dental implants, namely, general considerations, such as those related to biocompatibility, which apply to other kinds of dental materials which are to remain in the mouth for long periods of time, and specific considerations, such as those which affect osseointegration and inflammation, and dynamic fatigue.

11.6.1. Biocompatibility

Some informed advice on the biocompatibility testing of medical devices in general was recently published in two interesting articles by Lister. [104, 105].

Dental restorative materials could cause a number of adverse effects, so the strategic use of a number of different tests may be necessary. Currently, the aim is to avoid animal tests wherever possible, whilst recognising that *in vitro* test procedures need to take full account of the specific clinical situations in which the materials will be used. As Schmalz has pointed out, this can be achieved, for example, by including suitable barriers between the material and the target cells, by using appropriate target cells, and by choosing clinically relevant markers for measuring the biological effects caused by the material. [106].

These issues are taken into account in *ISO/FDIS 7405:2008*, which is concerned with evaluation of the biocompatibility of medical devices used in dentistry. [107] It should be used in conjunction with *ISO 10993-1:2009*, [89] and, in particular with *ISO 10993-2:2006*. [95] There are specific references to implant devices and, in particular, to endosteal implants.

The types of tests to be considered are divided into three groups (Table 11-3): Group I – cytotoxicity tests; Group II – animal tests; and Group III – specific tests for medical devices used in dentistry. Annex A of the guidance indicates that all the types of test should be considered for implant devices, except the pulp and dentine usage test. The animal tests to be considered would presumably be conducted according to the OECD TG. It is noted in Annex C that the classical LD50 test for acute toxicity has “been determined to be unnecessary and unacceptable on animal welfare grounds”, and has been superseded by other procedures.

Table 11.3. Biocompatibility tests for medical devices used in dentistry [105]

Group I: cytotoxicity tests	Group II: animal tests for:	Group III: tests specifically for medical devices used in dentistry
• Agar diffusion test • Filter diffusion test • Direct contact or extract tests • Dentine barrier cytotoxicity test • Tooth slice model	• Acute systemic toxicity (oral application) • Acute systemic toxicity (exposure by inhalation) • Subacute and subchronic systemic toxicity (oral application) • Skin irritation and intracutaneous reactivity • Delayed-type hypersensitivity • Genotoxicity • Local effects after implantation	• Pulp and dentine usage test • Pulp capping test • Endodontic usage test

The issues raised by the biocompatibility testing of dental restorative materials have been the subject of many reviews, including that by Murray *et al.* [108] They noted that dogs, ferrets and non-human primates have been used to investigate the responses of teeth to restorative treatments, but that it is not possible to ascertain the numbers of animals used for this purpose. They conclude that “the ideal approach for biocompatibility testing is to test solely *in vivo* with human subjects”, but that this is problematic, because of legal and ethical considerations. Nevertheless, the US Public Health Service has published a useful rating system, with an alpha (success) to delta (failure) scale, for use when materials are placed in patients following institutional review board approval and patient informed consent.

11.6.2. Osseointegration

Peri-implant endosseous healing, reviewed in detail by Davies, [109] is a vital part of the osseointegration of dental implants, where migration of osteogenic cells to the implant surface is followed by *de novo* bone formation, then bone remodelling. The surface of the implant can have a significant effect *in vivo* and *in vitro*, [110, 111] as can the surface area of the bone–implant contact, and pores in the implant can provide routes of entry for osteoblasts. This can lead to the fusion of the implant with bone, with no intervening connective tissue cells. There is evidence that the absorption of selected growth factors onto implants can stimulate and improve bone healing, [111] and tissue engineering for bone repair promises a way forward in cases of defective healing. [112].

11.6.3. Other Issues

The long-term culture of osteoblasts in a bioreactor can be used to reproduce the full process of osteogenesis *in vitro*. [113] This could provide an approach to studying the essentials of the microenvironment involved in osseointegration, and the basis for tests on the

wider efficacy and safety issues involved in short-term and medium-term interactions between dental implants and healing bone.

Other questions are related to the ability of the endosseous implant to provide long-term support for the crown or bridge to be built on it, which involves considerations of functional loading and fatigue. *ISO 14801:2007* specifies a method of fatigue testing of single post endosseous dental implants of the transmucosal type and their pre-manufactured prosthetic components, and can be used to compare implants of different designs or sizes under “worst case” conditions. [114].

11.6.4. Conclusion

The safety concerns related to dental implants are on an entirely different scale from those related to many types of pharmaceuticals, such as antineoplastics, antidepressants, anxiolytics, antibiotics and NSAIDs, many of which have had to be withdrawn because of adverse side-effects. The materials used in dental implants are comparatively inert, and the number of patients involved is likely to be comparatively small, and their identities will be known and recorded. As a result, an effective system of post-implantation follow-up and post-marketing surveillance, perhaps associated with the use of pre-operational biomarkers or non-invasive tests to predict patient compatibility with the implant, rather than implant compatibility with the patient, should be all that is needed. The case for conducting routine studies in animals cannot be a strong one.

Conclusion

Having regional and international agreements on the laws, regulations, guidelines and procedures involved in evaluating chemicals and chemical products of many kinds is only part of the equation — it is their interpretation and application which determines what is actually done. As a result, those who manufacture and market chemicals and chemical products are frequently faced with a variety of hurdles to be overcome, at home and in other parts of the world.

These hurdles, which can reflect nationalism, protectionism and social differences, also influence when it can be considered necessary and justifiable to conduct laboratory animal tests, what tests should be conducted, whether non-animal test procedures are acceptable, and how the results obtained should be evaluated, and the purposes for which they could meaningfully be applied.

Whether or not a particular test or testing strategy is reliable and relevant for its stated purpose must be the inescapable ethical and scientific basis for the establishment of its necessity. This clearly applies to animal testing, where the morality of causing animal suffering is debatable, even when the justification for it can be considered to be strong. It should not be permissible to conduct such tests when they cannot be specifically justified on a case-by-case basis — routine compliance with an established guideline, adopting a mindless check-list approach, bowing to the demands of one particular country or region, or

succumbing to the whims of an individual regulator, can provide no defence against criticism on ethical grounds.

This means that there must be an ongoing and endless review of all the procedures that are being developed, validated, accepted and applied — and, most of all, perhaps, what is demanded by the regulatory authorities. One of the better aspects of the new EU Directive on animal experimentation [71] is that the Member States must consider whether there should be a retrospective assessment of a project, taking into account whether its objectives were achieved, the harm inflicted on the animals, and what was done to implement the Three Rs. If such assessments were independently and objectively conducted, they would represent a challenge which many of the routine animal test procedures, including those for carcinogenicity and reproductive toxicity, and those which fail to identify the adverse effects later encountered when new drugs are given to patients, just could not meet.

Finally, however, it must be admitted that the move toward non-animal tests and testing strategies, which should be promoted and accelerated as much as possible, is not a way of escaping altogether from ethical and scientific dilemmas. One problem will be choosing a logical and intelligent pathway through an enormous number of possibilities, which are continually being elaborated and complicated as new developments take place. The best way of answering a question today will almost certainly not be the best way of answering it tomorrow, next week or next year. The use of integrated and intelligent testing strategies in the prediction of toxic hazard will be essential. [115] The new technologies undoubtedly offer many exciting possibilities — but are we good enough to truly reap their benefits, in the interests of both human and animal welfare?

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Part V – Methods for Bone-Implant Interface Analysis

Chapter XII

Histological Methods for Analyzing a Bone-Implant Interface

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Abstract

In the field of dental implantology, the interaction of bone and dental implant is one of the most important factors for long-term implant stability. Although many non-invasive methods, such as micro-computed tomography, have been developed to observe the 3-dimensional structures of these tissues, histological observation remains the gold-standard methodology to observe the bone-implant interface. Histology can provide unique information on cellularity and dynamic indices of bone remodeling. In this chapter, we summarize both the theoretical principles and practical techniques in histology for analyzing the bone-implant interface. We give a brief review associated with aspects of non-decalcified tissue preparation, immunohistochemistry, fluorescence microscopy, and quantitative microscopy with a view to motivating histological studies in this field.

12.1. Introduction

Histology is the study of the microscopic anatomy of cells and tissues of plants and animals. Histology is an essential tool in the field of biology and medicine. It is performed by examining a thin slice (section) of tissue under a light microscope, electron microscope or fluorescence microscope. Histology is composed of several basic steps: fixation, decalcification, dehydration, embedding, sectioning, staining, observation and the qualitative/quantitative analysis. In the field of dental implant research, implants and

surrounding tissue, such as the bone and gingival, are the main focuses. Although many non-invasive methods, such as micro-computed tomography, have been developed to observe the 3-dimensional structures of these tissues, histological observation remains the gold standard in these studies.

The histological process of tissues can be in both the non-decalcified and decalcified form. Decalcification will remove calcium deposits in tissues, and this is essential for a good embedding procedure in paraffin. The decalcified tissue is much more favorable for immunochemical staining than the non-decalcified tissue. [1] On the other hand, non-decalcified sections could preserve the mineral phase within the bone and other calcified tissues. This is a prerequisite for staining methods suitable for the delineation of various calcified tissue compartments. Only a non-decalcified tissue preparation can be applied to dental metallic implants. Consequently, in this chapter, we mainly focus on non-decalcified tissue preparation for observing the bone-implant interface.

Immunochemical staining refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. Immunochemical staining is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Light microscopy and fluorescence microscopy are the main observational methods for qualitative and quantitative analysis. A fluorescence microscope is an optical microscope used to study the properties of organic or inorganic substances using the phenomena of fluorescence and phosphorescence instead of, or in addition to, reflection and absorption. A quantitative analysis can be performed with a science known as stereology. Stereology was originally defined as "the spatial interpretation of sections". It is an interdisciplinary field that is largely concerned with the three-dimensional interpretation of planar sections of materials or tissues. It provides practical techniques for extracting quantitative information from three-dimensional material from measurements made on two-dimensional planar sections of the material.

In this chapter, we introduce basic knowledge and practical methods about the histological process for analyzing the bone-implant interface.

12.2. Non-Decalcified Tissue Preparation

12.2.1. Fixation of Tissue

Fixation is the permanent preservation of tissues in as life-like a state as possible. Fixation should be carried out as soon as possible after removal of the tissues (in the case of surgical pathology) or after death (with autopsy) to prevent autolysis and to reduce possible infectivity. Various histological fixation methods can be successfully applied to calcified specimens. They should fulfill 3 main conditions: 1) no reaction with the mineral phase; i.e. neither decalcification (low pH or chelation) nor precipitation of calcium phosphate; 2) fast and thorough penetration; and 3) good extraction of lipids and fat from the bone and bone marrow.

Five main categories of fixatives are available: 1) aldehydes; 2) mercurials; 3) alcohols; 4) oxidizing agents; and 5) picrates.

12.2.1.1. Aldehydes

Formaldehyde and Glutaraldehyde were recommended for light microscopic examinations by Horton et al. [2] They are the most commonly used aldehyde fixatives. Tissue is fixed by cross-linkages formed both within and between proteins, particularly between lysine residues.

Damage to the tertiary structure of the proteins from formaldehyde occurs on a limited scale, so that the antigenicity will not be lost. Therefore, formaldehyde is good for immunoperoxidase techniques. Formaldehyde penetrates tissues well, but it is relatively slow. Formalin (37% aqueous formaldehyde) is normally diluted 10 fold and neutrally buffered to make a working fixative solution of 4% formaldehyde. The neutralization of the acidity in formaldehyde with ammonia or PBS buffer is important in preventing autolysis and the development of tissue coloration caused by formalin pigment. On the other hand, formaldehyde is a suspect cancer hazard and a strong sensitizer. High exposures may be fatal. Formaldehyde can cause blindness if swallowed. Glutaraldehyde is corrosive, causing severe eye burns, skin irritation, respiratory tract and allergic reactions. The low cost of formaldehyde fixatives is one reason for their popularity.

Glutaraldehyde causes a deformation of the alpha-helix structure in proteins, limiting its usefulness as a fixative for immunological stains. However, it fixes very quickly so that it has become the standard fixative for electron microscopy. It penetrates very poorly, but gives the best overall cytoplasmic and nuclear detail. The standard solution is a 2% buffered glutaraldehyde. Recently, Glutaraldehyde has become identified as a powerful allergen, severely limiting the use of this fixative.

12.2.1.2. Mercurials

Mercurials contain mercuric chloride (HgCl_2) with an unknown mechanism of fixation. These fixatives penetrate relatively poorly and cause some tissue hardness. However, they are fast and give excellent nuclear detail. Their best application is for the fixation of hematopoietic and reticuloendothelial tissues. Since they contain mercury, they must be disposed of carefully. Mercuric chloride is fatal if swallowed. Mercuric chloride causes severe irritation to the eyes, skin, and respiratory tract.

12.2.1.3. Alcohols

Alcohols, including methyl alcohol (methanol) and ethyl alcohol (ethanol), are protein denaturants and are very good for cytologic smears because they act quickly and give good nuclear detail. Nonetheless, they are not routinely used for tissues because they cause too much brittleness and hardness.

12.2.1.4. Oxidizing Agents

Oxidizing agents include permanganate fixatives (potassium permanganate), dichromate fixatives (potassium dichromate), and osmium tetroxide. They are powerful denaturants and are, therefore, of limited use. Potassium permanganate, potassium dichromate and osmium tetroxide are strong oxidizers and can cause severe burns to any area of contact, possibly fatal if swallowed or inhaled. Furthermore, potassium dichromate is a known carcinogen.

12.2.1.5. *Picrates*

Picrates include fixatives with picric acid that have an unknown mechanism of action. It does almost as well as mercurials with nuclear detail but does not cause as much hardness. Picric acid is an explosion hazard in its dry form. As a solution, it stains everything it touches yellow, including skin.

Important considerations for the fixation of bone implant specimens are:

- 1) Penetration speed: For large tissue samples, the rate of penetration of the fixative into the sample must also be considered. Formalin and alcohol penetrate the fastest, glutaraldehyde the slowest, with mercurials falling between these two extremes. Although microwaves or relatively higher temperatures can dramatically improve the speed of fixation, they may also lead to a denaturation of proteins and, thus, compromise the immunological staining.
- 2) The amount of fixatives: The standard ratio of fixative to tissue volume is 10:1 (V:V). Lower volumes can be used if frequent changes of the fixative are carried out. Agitation will enhance the process.
- 3) Observation method: If immunological detection methods are to be used, a fixative that preserves protein structure is required.
- 4) Concentration of fixatives: The concentration of the fixative can affect the rate of fixation and the total penetration of fixative into the sample. Too high a concentration will lead to a hardening of the tissue and the formation of excessive artifacts. Too low a concentration will allow for the exhaustion of the fixative before the process is complete.
- 5) Safety of fixatives: Fixatives are among the most hazardous substances used in life science research. Work with these substances must be under the hood, wearing gloves, a lab coat and safety goggles.
- 6) Safe disposal of aldehyde waste: The disposal of these fixatives should comply with local regulations.
- 7) The price of a fixative may also be a factor.

12.2.2. Dehydration and Clearing

Dehydration and clearing for non-decalcified tissue do not differ from general histology. Samples are transferred through baths of progressively more concentrated ethanol to remove the water (Table 12–1). The term "clearing" comes from the fact that the clearing agents often have the same refractive index as proteins. As a result, when the tissue is completely infiltrated with the clearing agent, it becomes translucent. This change in appearance is often used as an indication of the effectiveness or completeness of the clearing process. Six kinds of clearing solutions are available: 1) xylene; 2) toluene; 3) chloroform; 4) methyl salicylate; and 5) orange oil.

The most common clearing agent is xylene. Xylene is reasonably cost effective and works well for short-term clearing of small tissue blocks. Long-term immersion of tissue in xylene results in tissue distortions. Toluene is better at preserving the tissue structure and is more tolerant of small amounts of water left behind in the tissues than xylene is.

Table 12.1. A conventional protocol for embedding non-decalcified tissues in methyl methacrylate (MMA)

Event	Chemicals	Duration	Temperature
Fixation	10% Formalin	>1week	RT
Rinsing	Tap water rinsing	Overnight	RT
Dehydration	70% ETOH	2 days	RT
	80% ETOH	2 days	RT
	95% ETOH	2 days	RT
	100% Ethanol I	2 days	RT
	100% Ethanol II	2 days	RT
	100% Ethanol III	2 days	RT
Clearing	Xylene I	2 days	RT
	Xylene II	2 days	RT
Infiltration	MMA+15% Dibutylphtalate	2 days	RT
	MMA+15% Dibutylphtalate +0.5% perkadox	2 days	4°C
Embedding	MMA+15% Dibutylphtalate +1% perkadox	until hardening	12°C
	Oven	Overnight	60°C
	MMA: methylmethacrylate (MERCK, 8.00590.2500)		RT: room temperature
Notes:	Dibutylphthalate (MERCK, 8.00919.2500)		
	Perkadox (Sigma, 40451)		

However, toluene is more expensive than xylene and more toxic, so toluene is less commonly used. Chloroform has been used in some applications, but it is a severe health hazard, acts slowly and may lead to sectioning difficulties. Methyl salicylate is safe and effective, though rarely used due to the cost. Clearing agents based on orange oil, such as National Diagnostics Histo-Clear, offer the best clearing action with the lowest hazard rating of all the xylene alternatives. Histo-Clear is excellent for preserving fine tissue structure, and can often be used instead of xylene with no alteration of protocol. A thorough infiltration and substitution of the media is essential. For this reason, enough time should be allowed for each single step of dehydration and clearing; and solutions should be regularly replaced.

12.2.3. Embedding of Non-decalcified Specimens

12.2.3.1. Embedding Medium

A variety of plastic materials have been tested for the plastic embedding of non-decalcified specimens. Methacrylates are recommended for light microscopy. Methacrylates are the basis of different mixtures now in use in many laboratories. Pure methyl methacrylate (MMA) is hard and brittle, and it is only suitable for saw cuts and ground sections. The addition of a softener such as polyethylene glycol, dibutyl phthalate, or plastoid N to methyl methacrylate turned out to be a better solution for adjusting the consistency of the blocks [3].

MMA embedding of non-decalcified bone biopsies is a technique widely used for bone histomorphometry and bone marrow hematopathology, both in clinical medicine and in animal experimental studies. [4] Compared with other plastic embedding media such as water-soluble methacrylates (e.g., glycolmethacrylate, GMA), MMA offers a variety of advantages: 1) MMA penetrates tissues better than GMA. This is of importance especially for the larger bone specimens such as rat tibiae or vertebrate or human trans-iliac biopsies, and the histological quality of bone sections is generally higher in MMA-embedded bone samples compared to GMA-embedded specimens; 2) GMA is a bi-functional molecule and forms a crosslink during polymerization of the plastic. Therefore, removal of the resin from tissue sections is not possible for GMA-embedded material. MMA, on the other hand, can easily and completely be removed from the tissue sections. This results in superior staining characteristics and excellent morphological detail.

A MMA mixture which penetrates rapidly and polymerizes even at 4°C was previously described. [5] These low temperatures are a prerequisite for histochemical reactions, especially for the demonstration of acid phosphatase in osteoclasts and alkaline phosphatase in osteoblasts.

12.2.3.2. Conventional Protocol of Embedding in MMA

Here, we recommend the protocol (Table 12–1) that is used in our lab for embedding non-decalcified tissues. The quality of the tissues that are treated with this standard protocol is already sufficient for the chemical surface-staining of bone, bone marrow and biomaterials as well as for the histochemical staining of tartrate-resistant acid phosphatase in osteoclasts. [6] However, conventional MMA embedding causes almost a complete loss of enzyme activity and protein antigenicity in the tissues, precluding its use for immunochemistry.

12.2.3.3. Special Protocol for Embedding in MMA for Immunohistochemical Staining

Erben reported a modified embedding technique (Table 12–2) which employed super low temperatures to maximally preserve enzyme activity and antigenic determinants in bone tissue. [7] Consequently, the technique is suitable for histochemical and immunohistological applications as well as for routine bone histomorphometry. In this protocol, the addition of methyl alcohol, during the infiltration and polymerization of the plastic, significantly improved the maintenance of tissue antigenicity.

The staining of alkaline phosphatase and tartrate-resistant acid phosphatase, as well as the positive immunohistochemical staining of Kupffer cells and lymphocytes with the monoclonal antibody ED1 in sections of liver, tibiae, and vertebrae of 3-month-old rats, could be obtained through this technique. The method described here might be useful for the inclusion of immunohistological methods into bone histomorphometry.

Technovit® 9100 is a commercially available kit for such staining using the same principle. [8] The chemical polymerization of Technovit 9100 takes place under the exclusion of oxygen, with the aid of a catalytic system consisting of peroxide and amine. Additional components such as PMMA-powder and stabilizer allow for a controlled polymerization at a low temperature (-2°C to -20°C), guaranteeing a complete conduction of polymerization heat. The polymerization time is, at these temperatures and a volume of 3 – 15 ml, approximately 18 – 24 hrs.

Table 12.2. A special protocol for embedding in MMA for immunochemitric staining

Event	Chemicals	Duration	Temperature
Fixation	Fixation with 10% Paraformaldehyde in 0.1M phosphate buffere, at pH 7.4	1 day	4°C
Rinsing	rinsing with 0.1M phosphate buffere, at pH 7.4, containing 10% sucrose	overnight	4°C
Dehydration	70% ETOH	2 days	4°C
	95% ETOH	2 days	4°C
	100% 2-propanol	twice for 1 day	4°C
Clearing	Xylol I	2 days	4°C
	Xylol II	2 days	4°C
Infiltration	MMA I	3 days	4°C
	MMA II	3 days	4°C
	MMA III	3 days	4°C
Embedding	Polymerization MMA exclude air	< 3 days	-20°C
Formula	MMA I: 60 ml MMA (Merck; containing 100 ppm hydrochinon)+35ml butylmethacrylate (Sigma; containing 10 ppm hydrochinon)+15 ml methylbenzoate +1.2 ml polyethylene glycol 400. MMA II: 100 ml MMA I with 0.4 g dry benzoyl peroxide. MMA III: 100 ml MMA I + 0.8 g dry benzoyl peroxide. Polymerization MMA: 400 ml of N,N-dimethyl-p-toluidine + 100 ml of cold (4°C) MMA III		

12.2.4. Sectioning

12.2.4.1. Microtome

A microtome is a sectioning instrument that allows for the cutting of extremely thin slices of material, known as sections. Three main microtomes are commonly used in the preparation of non-decalcified tissue in the field of oral implantology: 1) rotary microtomes, 2) sledge microtomes, and 3) saw microtomes.

12.2.4.2. Rotary Microtomes

This instrument is a common microtome design and operates with a staged rotary action such that the actual cutting is part of the rotary motion. In a rotary microtome, the knife is typically fixed in a horizontal position. The rotary microtomes come with additional (1130) or inbuilt motor drive (1140 Autocut). Various knife carriers and block holders are available. Rotary microtomes offer sufficient stability for cutting blocks up to 20-25mm in diameter or, referring to the tissue size, areas with a smaller side length of 10-15mm. This is sufficient for metaphyseal bones of smaller animals, transverse sections and biopsies of the human iliac crest, or even the intermediate and distal phalanges of human fingers. Larger amounts of cortical bone within the section may reduce the overall size that can be readily cut. The typical cut thickness for a rotary microtome is between 1 and 60 µm. For hard materials, such as a sample embedded in a synthetic resin, this design of microtome can give good "Semi-thin" sections with a thickness as low as 0.5 µm.

12.2.4.3. Sledge Microtome

A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), then moving backwards and forwards across a knife. Modern sledge microtomes have the sledge placed on a linear bearing, a design that allows for the microtome to readily cut many coarse sections. By adjusting the angles between the sample and the microtome knife, the pressure applied to the sample during the cut can be reduced. Typical applications for this design of microtome include preparing large samples such as those embedded in paraffin for biological preparations. The typical cut thickness on a sledge microtome is between 1 and 60 μm . This kind of microtome can be used to cut larger specimens (e.g. human femoral heads). The stability of these larger types depends not only on the weight and rigidity of their construction, but also on the design of the specimen holder, excluding any adjustment or positioning of the block. This reduces its versatility and sometimes it necessitates a tedious trimming and shaping until the sectional plane is finally oriented correctly.

12.2.4.4. Saw Microtome

The saw microtome is especially used for hard materials such as teeth or bones. The microtome of this type has a recessed rotating saw that slices through the sample. The minimum cut thickness is approximately 30 μm , and it can be made for comparatively large samples.

Industrial grade diamond knives are used to slice hard materials such as bone, teeth and implant materials. For example, the Leica SP1600 saw microtome (Figure 12–1) is specially designed for cutting extremely hard and brittle industrial materials embedded in methylmethacrylate with a maximum size of 35 mm in diameter.



Figure 12.1. A saw microtome which is equipped with a diamond saw blade.

The Leica SP1600 saw microtome works with different materials ranging from decalcified or non-decalcified bone with implants embedded in methylmethacrylate; teeth; mineralogical samples to all kinds of industrial materials such as steel, titanium or bioceramics.

12.2.4.5. Section Cutting and Mounting Using Saw Microtome

The final quality of the sections depends greatly on the practical operation. The steps and important tips for the operation of rotary and sledge microtomes have already been previously described. [4] Therefore, in this section, we focus mainly on the operation of the saw microtome, which can be used to cut the samples with metallic implants, for example by using the Leica microtome SP1600.

Mounting Samples to Sample Holder

The mounting of a sample is a very important step in securing the stability of the sample during cutting. Any instability of a sample may severely damage the saw blade during cutting. The stage area varies with the type of saw microtome and mostly lies between 6 and 30 mm in diameter. Samples with sizes within this range can be mounted directly and secured with the knurled wheel. The contacting surface between the samples and the stage should be sufficient to ensure adequate bonding strength. Smaller or larger samples are first cemented to plates with an adhesive. The quality of adhesive is very important, we suggested using super-adhesive and water-proof glue, with either 1 or 2-components. At least 5 minutes should be allowed for adequate bonding. The oxidation may significantly compromise the efficiency of glues. Therefore, the glue should be kept sealed whenever possible. If many samples are to be sawed, a MMA table (5-1mm) can be used to avoid frequent cleaning and possible damage to the table. However, the MMA table should be reasonably thin; otherwise the cutting range of the samples may be significantly compromised.

Setting the Height of the Sample

Setting the sample holder until the surface of the sample is slightly above the upper edge of the saw. Tighten the screw for fixation.

Trimming the Sample Surface

To avoid damage from overheating, a sufficient spray of tap water should be given to the contact point between the saw blade and the sample. Turn on the motor with the switch and release the sample towards saw blade. To save time, the speed of the sample feed can be increased while the sample is guided towards the edge of the blade. Then turn to a low speed.

The sample should meet the saw blade at this low speed. Shortly before contact is made, turn back to the speed necessary for sawing. To avoid any possible artifacts and deep scratches, the saw blade speed to the sample should be kept in a low range (0-10 for the Leica saw SP1600). Caution should be taken especially when some materials with a high hardness, such as ZrO_2 , are included. The figures engraved on the knurled knob are not absolute speed values, but only guidelines to enable repeat settings.

The most favorable feed rate must be determined for each individual sample. A general rule applies: the lower the speed, the less the forces coming to bear on sample and saw blade will be, i. e. the gentler the sawing process will be.

Making the Sections

After the sample has passed through the saw, turn the sample arm back as far as it will go. It clicks into place audibly. Turn off the motor and remove the section from the blade.

Setting the Section Thickness

When setting the section thickness, the thickness of the saw blade must always be taken into account. For example, a setting of 400 μm is necessary to obtain a 100 μm thick section if a 300 μm saw blade is used.

Removing the Section

If the section is relatively thin, it will stick to the blade after sawing because of the adhesive power of the water. Thicker sections are generally pushed to the outer edge of the saw blade by the centrifugal force. Switch off the motor and remove the section. It can happen, although it is very unusual, that the section falls inside the microtome. It should not be recovered before the sectioning work has been completed because the sample holder and saw have to be removed.

Changing the Sample

Push the object arm back until it clicks into place. Switch off the motor and turn off the water supply. As we recommend, you can simply cut off a thin layer of MMA table to obtain a vertical plane to the saw blade for next sample.

12.2.5. Mounting, Grinding and Polishing

The sections can be mounted on a semitransparent Plexiglas plate (Figure 12–2) or a transparent glass slide with glue. A rough surface is needed to ensure the adhesion of the sections onto the holders. Air bubbles should be avoided under the sections. The sections can be ground and polished with the section holders by using sand paper with sequential grits. It is suggested to do the grinding and polishing on an automatic rotary machine.

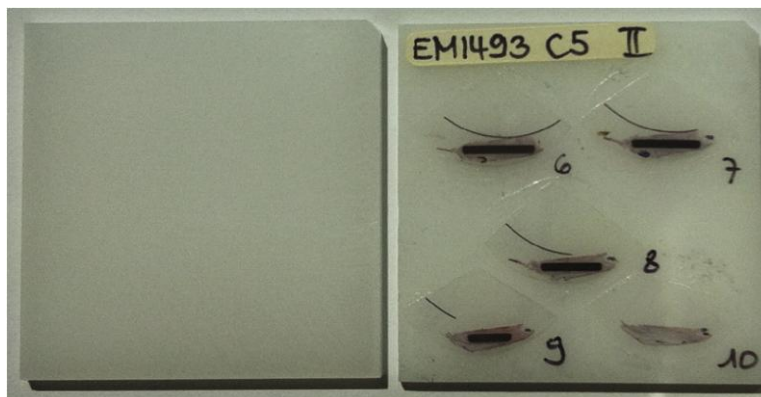


Figure 12.2. Semitransparent Plexiglas plates without (left) or with mounted sections (right).

The selection of the grit of sand papers can be as following: P400, P1000, P2500 and finally with P4000 for polishing (P: ISO/FEPA Grit designation). Polishing with P4000 can take longer to completely remove any visible scratches on the sections. Caution must be taken when using P400 sand papers since it can remove MMA very soon. A frequent checking is needed. The slides can be ground to any thickness. The thin sections ($<15\mu\text{m}$) can be used for immunochemical staining and fluorescence observation whereas the sections ($>100\mu\text{m}$) can be used for surface staining.

12.2.6. Surface Staining

Biological tissue has little inherent contrast with either a light or electron microscope. Staining is employed to give a strong contrast to the tissue in order to highlight the particular features of interest. Where the underlying mechanistic chemistry of staining is understood, the term histochemistry is used.

A large variety of staining methods are used to stain non-decalcified tissues. The methods for staining sections that are prepared by rotary microtome were previously described elsewhere. [4] In this section, we focus on the surface staining of MMA-embedded sections that are prepared with saw microtomes.

12.2.6.1. Removing MMA

MMA needs to be removed to allow for the infiltration of staining chemicals into the tissues. Other steps such as the transfer from Methyl cellosolve acetate (MCA) to the watery solutions, differentiation, dehydration, clearing and final mounting do not differ from general histology. Conventionally, for the surface-staining of MMA embedded sections containing bone and implants, we treat the sections with a 50% Ethanol bath in an ultrasonic condition for 30 minutes. $3\text{--}5\mu\text{m}$ of MMA will be removed, exposing the underlying mineralized and unmineralized tissues.

12.2.6.2. Staining

Bone, osteoid, cartilage, interconnective tissue and implanted biomaterials are the most frequently observed objectives in the field of implantology. In this section, we introduce two practical surface staining methods that are conventionally used in implant related research.

Mc Neal's Staining

This surface staining method — Mc Neal's staining — was recommended by professor Robert K. Schenck [4] and is still widely used for bone histomorphometry. [9, 10] The formula of staining solutions is described in detail in Table 12–3. With the prepared solutions, Mc Neal's Staining is performed according to the following protocol:

- 1) Immersion in Mc Neal's Working Solution for 10 minutes
- 2) Wash with tap water for 3 minutes and dry with tissue paper
- 3) Immersion in 0.1% Formic acid for 3 minutes
- 4) Wash with tap water for 3 minutes and dry with tissue paper
- 5) Immersion in Mc Neal's Working Solution for 10 minutes

- 6) Wash with tap water for 3 minutes and dry with tissue paper
- 7) Immersion in 0.1% Aqueous Basic Fuchsin for 30 to 60 seconds
- 8) Wash with tap water for 3 minutes and dry with tissue paper

After Mc Neal's staining, the new bone is reddish and the old bone is pinkish. Cells and nuclei are blue. Cartilage is purple. Connective tissue is pinkish and red. In figure 12–3, we show an example of Mc Neal's staining on a cross-section through a titanium disc that was functionalized by a BMP-2-incorporated biomimetic calcium phosphate coating. The bone is reddish and nuclei are blue. Connective tissues are pink, and fat tissues are white.

Table 12.3. Formula for preparing a solution for Mc Neal's staining

I. Stock Solution of Modified Mc Neal's Tetrachrome		
Chemicals	Manufacture	Quantity
Methylene Blue Chloride	Sigma M9140	1.0 grams
Azure A Eosinate	Sigma 200212	1.6 grams
Methyl Violet, Bernthsim	Sigma 198099	0.2 gram
Methyl Alcohol		500 ml
Glycerin		500 ml
Heat at 50°C for 1 day, then 37°C for 3 days in oven (capped). Filter and store in dark bottle in dark.		
II. 0.1% Basic Fuchsin		
Chemicals	Manufacture	Quantity
Basic Fuchsin		0.5 grams
Deionized water		500.0 ml
Stir on Stir plate and filter afterwards		
III. 0.1% Formic Acid		
Chemicals	Manufacture	Quantity
Formic Acid	Sigma 33015	1.1 ml
Deionized water		1000.0 ml
Stir on Stir plate and filter afterwards		
IV. 0.1% Toluidine Blue		
Chemicals	Manufacture	Quantity
Toluidine Blue O	Sigma 198161	1.0 ml
Deionized water		1000.0 ml
Stir on Stir plate and filter afterwards		
V. Working Solution of Modified Mc Neal's Tetrachrome		
Chemicals	Manufacture	Quantity
10% Mod. Mc Neal's (Stock Solution)		5 ml
0.1% Toluidine Blue		2.5 ml
Distilled Water		42.5 ml
Stir on Stir plate and filter afterwards		

Tartrate-Resistant Acid Phosphatase Activity (TRAP)

Histochemical staining, such as TRAP staining, can also be performed on the MMA embedded sections. We also recommend here a protocol that has been used for TRAP staining. [11] The formula for preparing staining solutions is described in Table 12–4. With the prepared solutions, TRAP activity Staining is performed according to the following simple protocol:

- 1) Incubate 4 hours at 37 °C with a 0.5 ml/slice working solution
- 2) Wash with deionized H₂O

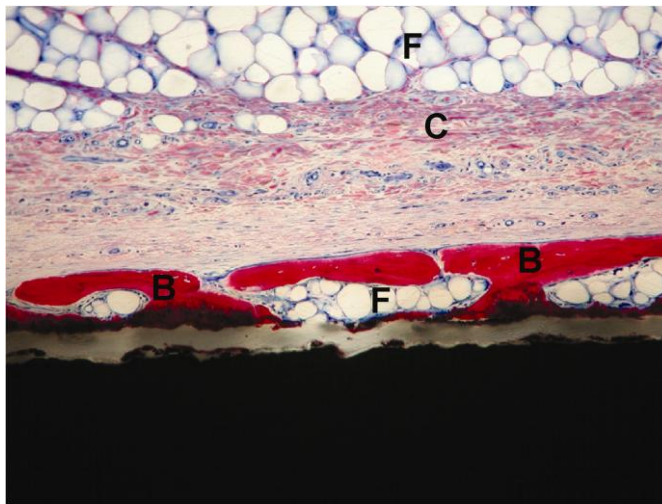


Figure 12.3. Light micrographs depicting a cross-section through a titanium disc that was functionalized by a BMP-2-incorporated biomimetic calcium phosphate coating and implanted subcutaneously in rats for 5 weeks. The section was stained with Mc Neal tetrachrome, toluidine blue O and basic fuchisine. Bone (B) is reddish and nuclei are blue. Connective tissues (C) are pink and fat tissues (F) are white.

Table 12.4. Formula for preparing a solution for TRAP staining

I Acetate buffer (0.2M):		
Chemicals	Manufacture	Quantity
100% Acetic Acid	Merck UN 2789	1.26 ml
Sodium Acetate anhydrous	Fluka 7179	6.51 grams
Deionized water		1000 ml
Stir on Stir plate and afterwards use 1N HCL or NaOH to adjust PH value to 5.2		
II. Working Solution (100ml)		
Chemicals	Manufacture	Quantity
Naphthol-AS-TR-phosphate	Fluka 70478	150 mg
N-N-Dimethylformamid	Fluka 71995	500 µl (use glass stick to grind till entirely dissolve)
Acetic acid buffer		100 ml
Na-Tartrat	Fluka 71995	150 mg (till entirely dissolve)
Fast Red Salt	Sigma F-8764	150 mg (mixed well)
Mixed and filtered		

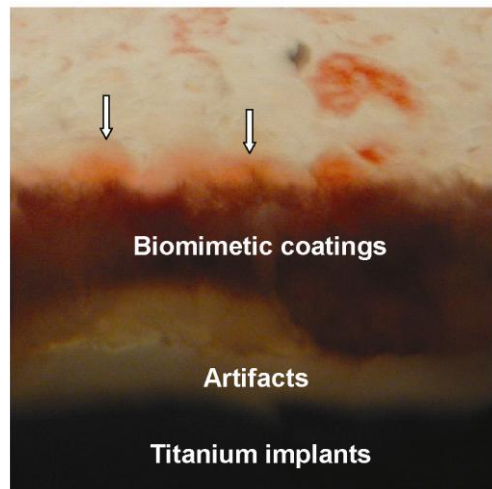


Figure 12.4. Light micrograph depicting a cross-section through titanium functionalized by a BMP-2-incorporated biomimetic calcium phosphate coating and implanted subcutaneously in rats for 5 weeks. The disc is artificially separated from its coating, which has an irregular thickness owing to its uneven resorption by the tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells are apparent at several locations (white arrows).

We show an example of TRAP staining on a cross-section through a titanium disc that was functionalized by a BMP-2-incorporated biomimetic calcium phosphate coating. TRAP staining positive cells present in orange (Figure 12-4).

12.3. Immunohistochemistry

The application of immunohistochemistry in the study of bone-implant interface is rarely reported. This is mainly due to the technical difficulties in preparing a plastic embedding that is suitable for immunohistochemistry. Technically, using immunohistochemistry to analyze the bone-implant interface should be based on the combination of low-temperature embedding (as we stated in section 2.3.3) and a cutting-grinding technique. Because of the inclusion of hard dental implants, the specimens can only be first cut into thick sections ($>100\mu\text{m}$) and then carefully ground to $3\text{--}10\mu\text{m}$ for immunohistochemistry. Although this technique is currently not widely used in the study of a bone-implant interface, it presents a promising technique for giving a deep insight into the interface at the level of molecular biology. In this section, we start with some basic concepts and principles of immunochemistry that can be used to analyze the bone-implant interface.

12.3.1. Concepts and Principles

Immunohistochemistry is the application of Antibody/Antigen interactions for providing information about biological systems. [12] Antibodies bind tightly and specifically to an "epitope" (one specific structure) on an "antigen" (foreign substance to the body). [13]

Antigens can be an enormous range of substances from simple chemicals, sugars, and small peptides to complex protein complexes such as bacteria, viruses and other infectious agents.

An antibody (immunoglobulin, Ig) is a glycoprotein in the immune system to identify and neutralize antigens. In mammals, there are five types of antibodies: Ig G, Ig A, Ig M, Ig E and Ig D, of which Ig G and Ig M are the most important for immunoassays. There are two classes of antibodies: polyclonal and monoclonal. Monoclonal antibodies are identical because they were produced by immune cells that are all clones of a single parent cell, whereas polyclonal antibodies are multiple antibodies produced by different types of immune cells that recognize the same antigen.

12.3.2. Antibody Labeling and the Choice of the Label

In order to visualize the antigen–antibody immunoreaction under the microscope, the antibody must be labeled with an easily detectable molecule, such as an enzyme, a fluorescent dye, colloidal gold or biotin. [14] Light microscopy primarily makes use of two detection systems: fluorescence and enzyme labeling.

12.3.2.1. Immunofluorescence

The conjugation of a fluorescent dye to the primary or secondary antibody allows its detection under ultraviolet light. Typically, Texas Red, Rhodamine, Fluorescein, or Phycoerythrin are conjugated to the antibody. Texas Red and Rhodamine fluoresce in the red, Fluorescein in the green and Phycoerythrin in the blue. This allows multiple label experiments, in which different antigens show up as different colors. While extremely sensitive, this technique suffers from the fact that the dyes fade with time.

12.3.2.2. Enzyme-Antibody Conjugate Methods

Enzyme labels are usually coupled to secondary antibodies or to avidin. The latter is used for the detection of biotinylated primary or secondary antibodies in ABC methods. [15] The enzyme labels routinely used in immunohistochemistry are horseradish peroxidase (HRP) and calf intestinal alkaline phosphatase (AP). HRP is often conjugated to antibodies, producing a system capable of detecting antigens by peroxidase staining. The incubation of HRP in a solution containing hydrogen peroxide and Diamino Benzidine (DAB) results in the reduction of DAB to an insoluble brown precipitate, visible under the light microscope. Alkaline phosphatase (AP) is also used on the same principle with a phosphorylated naphthol as its substrate. The dephosphorylated naphthol reacts with an azo dye such as Fast Red TR, to give a colored reaction product. A level of signal amplification is provided by the peroxidase-antiperoxidase complex system (PAP). This technique uses large complexes which contain many detection agents bound together. [16] Either of these techniques may be combined with the Avidin-Biotin system to provide a more robust and a more easily generalizable detection method. Biotin is a small molecule which is bound tightly and specifically by the protein avidin. This binding has been exploited by attaching biotin to the secondary antibody (biotinylation), and avidin to the detection enzyme.

12.3.3. Probes Processing in Immunohistochemistry

Most steps of the immunohistochemistry on non-decalcified sections are similar to those on decalcified sections, except for a step named “deplasticization”. Each step will have an impact on the quality of immunohistochemical staining, including fixation, washing and incubation conditions as well as on the appropriate mounting of cell and tissue specimens onto slides. Most of the protocols used in immunohistochemistry are, however, far from being standardized.

12.3.3.1. *Deplasticization and Redehydration*

Many protocols were developed to remove MMA from sections. It can be carried out by successive incubations in xylene (1 hr), 2-methoxyethylacetate (1 hr), and acetone (10 min). [17] An alternative using twice xylene for 20 min, twice 2-methoxyethylacetate for 20 min, twice acetone for 5 min, and 80% ethanol. [18] This step is crucial for an appropriate immunohistochemical labeling of hard tissue utilizing the Technovit[®] embedding medium, since the thorough removal of the embedding material from within the bone tissue will assure the antibody penetration, thereby allowing the antigen-antibody reaction. [19] Failure in the removal of embedding material prevents this reaction from occurring and would characterize processing failure. After deplasticization, endogenous peroxidase should be blocked with H₂O₂ and then rinsed in saline. [18] The subsequent steps of immunohistochemistry on non-decalcified sections are the same as those of decalcified sections.

12.3.3.2. *Antigen Retrieval*

Routine formaldehyde fixation and the rest of the procedure for paraffin embedding may alter the conformation of protein macromolecules, negatively affecting antigen and antibody binding and, consequently, decreasing the intensity of the final reaction in the immunohistochemical procedure. However, these disadvantages can be overcome by employing an appropriate antigen retrieval method such as the heat-induced antigen retrieval methods. These could be such as in the citrate buffer or the Ethylenediaminetetraacetic acid (EDTA) retrieval buffer overnight in a 60° oven or in 100° boiling buffer, as well the proteolytic antigen retrieval method. The proteinase K can also be used. [20, 21]

12.3.3.3. *Buffers for Washing and Antibody Dilution*

The type of buffer selected for washing and diluting antibodies is very important for obtaining a good immunohistochemical staining result. Buffers near physiological pH are routinely used. Because this pH lies within the range of isoelectric points of immunoglobins, the optimal immunoreactivity of antibodies is generally expected at pH 7.4. [22] Immunohistochemical staining is usually carried out with antibodies dissolved in a phosphate buffered saline (PBS) or in a Tris-buffered saline (TBS). When working with alkaline phosphatase-conjugated antibodies, TBS is the only alternative. The buffer should be freshly made to avoid the influence of bacterial contamination on the quality of the staining.

12.3.3.4. *Working with Antibodies*

Two principal immunohistochemistry methods are employed: direct and indirect (figure 12–5). The direct method is a straightforward one-step process that creates a direct reaction

between the antigen and the labeled antibody. Common labels of first antibody include fluorescent dye, biotin or enzymes. The direct method is applicable when using antibodies with high avidity and for localizing high density antigens ($>10^6$ molecules/cell). To localize antigens expressed at a lower level, the indirect method is affordable, requiring the use of two antibodies: a primary unlabeled antibody and a secondary labeled antibody.

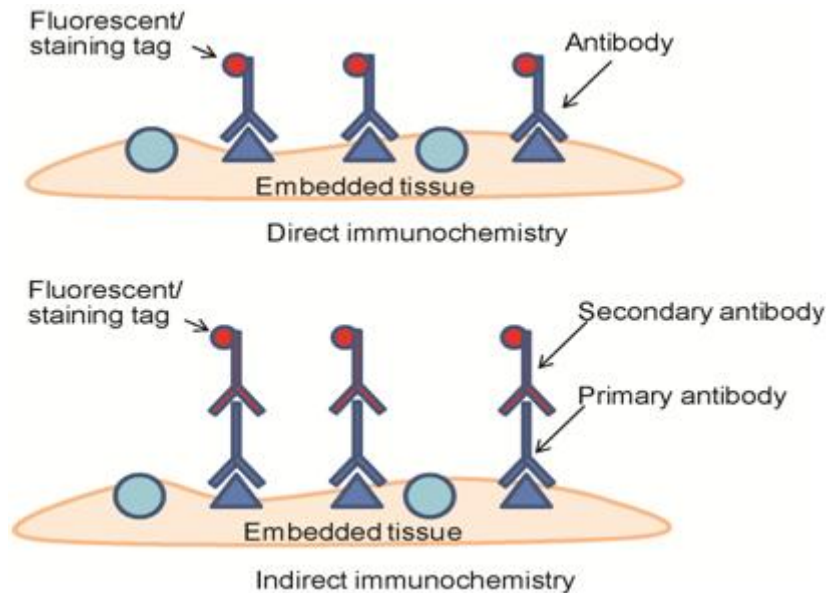


Figure 12.5. Schematic graph depicting the interaction of antigen and antibody in direct and indirect immunocytochemistry.

The Choice of Primary Antibodies

Monoclonal antibodies commonly produced by a mouse are highly specific. However, polyclonal antibodies produced by rabbits are known to have a higher affinity. [23] If possible, your primary antibody should not be raised in the same species as the tissue under study to avoid severe background staining.

The Choice of Secondary Antibodies

Caution should be exercised since secondary antibodies against immunoglobulins from one species may cross-react with a number of other species unless they have been specifically pre-absorbed against the cross-reacting species. Antibodies that have been pre-absorbed against other species are recommended when the possible presence of immunoglobulins from other species may lead to interfering cross-reactivity.

Optimal Concentration of the Antibody

The optimal working concentration of the primary antibody is determined by many factors, such as antigen density, loss of antigen activity in the course of specimen preparation, antibody avidity, and the sensitivity of the immunostaining procedure. In standard immunohistochemical procedures, the working concentration of the primary antibody lies within the range of 1 to 5mg/ml. Secondary antibodies are usually applied with the working

concentration of approximately 5 to 10mg/ml. The optimal working concentration can be determined in a series of stains with the antibody in several dilutions of two-fold increments.

12.4. Fluorescence Microscopy

A fluorescence microscope (Figure 12–6) is an optical microscope using the phenomena of fluorescence and phosphorescence to study the properties of organic or inorganic substances. Fluorescence is the emission of a portion of the energy absorbed by some molecules after a certain period of time (termed the fluorescence life-time) of absorbing the energy of a particular wavelength. The amount of energy emitted as fluorescence is always less than the amount of energy absorbed. Thus, the emitted light has a longer wavelength. A given molecule has its own characteristic wavelengths for excitation light and emission light. [24] The difference in the maximum wavelengths of absorption and emission is known as the “Stokes shift”.



Figure 12.6. A fluorescence microscope.

12.4.1. Basic Principles

All fluorescence microscopy methods share the same principle. [25] A specimen is illuminated with the light of a specific wavelength which causes fluorescence in the sample. The emitted light, which has a different wavelength from the excitation light, is then detected through a microscope objective. The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. The emission of light through the fluorescence process occurs almost simultaneously with the absorption of the excitation light because of the relatively short time delay between photon absorption and

emission. When emission persists 1 after the excitation light has been extinguished, then the phenomenon is referred to as phosphorescence.

Thus, fluorescence microscopes have the following four basic functions:

- 1) Generating excitation light to specimens; Excitation light sources and wavelength-selection devices allow the selection of the appropriate excitation wavelengths. Excitation light is delivered to the specimen through the microscope objective.
- 2) Separation of emission light from excitation light: A special mirror, known as a dichroic beam-splitting mirror, allows for the separation of the excitation light from the emitted fluorescence. The dichroic beam-splitting mirror has the special property of being able to reflect light below a specific wavelength, yet allow light above this specific wavelength to pass through the mirror unobstructed.
- 3) Collecting emitted light: Objective lenses have been developed that allow maximal collection of a portion of the emitted fluorescence from the sample; and
- 4) Optical observation.

Consequently, four typical components of a fluorescence microscope are a light source, the excitation filter, the dichroic mirror and the emission filter.

12.4.1.1. Excitation Light Source

There are a variety of excitation light sources that can be used for fluorescence microscopy. The choice of which light source to use will depend on the fluorescent probes being used. Because many of the available fluorophores require excitation in the blue/green portion of the visible light spectrum, mercury (Hg) (Figure 12–7), xenon (Xe), or Hg/Xe combination lamps are generally employed. Lasers can also be used.



Figure 12.7. An excitation light source.

Lasers offer monochromatic light of very high energies, and they can be used in continuous-wave or pulsed modes of operation. Lasers are commonly used in confocal microscopy. Please note that turning on the excitation light lamp should be at least 20 minutes after the last turning off.

12.4.1.2. Filters

An excitation filter ensures the illumination is nearly monochromatic and at the correct wavelength, whereas an emission filter ensures that none of the excitation light reaches the detector. The filters and the dichroic mirror are chosen to match the spectral excitation and emission characteristics of the fluorophore in the specimen.

12.4.2. Confocal Laser Scanning Microscopy (CLSM)

The development of CLSM enables us to obtain high-resolution optical images with depth selectivity. The key feature of confocal microscopy from normal fluorescence microscopy is its ability to focus images from selected depths, termed as optical sectioning. CLSM (Figure 12–8) can provide high-resolution images by scanning layer-by-layer. These can then be reconstructed by computers.

This feature of CLSM can also be used to acquire the 3-dimensional topography of non-opaque specimens and inspect the interior structure of opaque specimens. A conventional microscope "sees" as far into the specimen as the light can penetrate, whereas a confocal microscope only images one depth level at a time. In effect, the CLSM achieves a controlled and highly limited depth of focus.

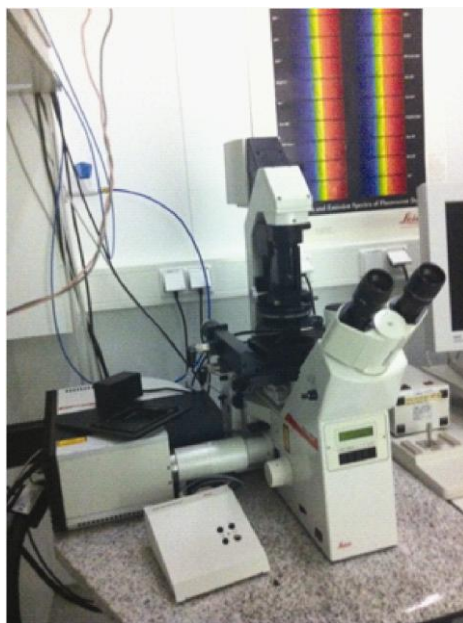


Figure 12.8. A confocal laser scanning microscope.

These characteristic features of CLSM are realized by a delicately designed optical pathway. A laser beam passes through a light source aperture which is then focused by an objective lens onto a small focal volume of a specimen. The emitted fluorescent lights from the focal volume, together with the scattered and reflected laser light, are then re-collected by the same objective lens. With a beam splitter, part of the light is separated off into the detection apparatus. After that, a pinhole will selectively pass the fluorescent wavelengths while blocking the original excitation wavelength. The light is then detected by a photodetection device, transforming the light into an electrical signal that is then recorded by a computer. [26].

12.4.3. Sample Preparation

There are three main methods of creating a fluorescent sample: 1) labeling with fluorescent stains; 2) biological samples expressing a fluorescent protein; and 3) auto-fluorescence.

12.4.3.1. *Fluorescent Stains*

Many fluorescent stains have been designed for a number of biological molecules. They are mainly divided into three categories:

- 1) Small molecules which are intrinsically fluorescent and bind a biological molecule of interest. For example, the DAPI (4',6-diamidino-2-phenylindole) and Hoescht can be used to stain nucleic acid.
- 2) Some drugs or toxins which bind specific cellular structures have been attached to a fluorescent reporter. For instance, fluorescent labeled phalloidin can be used to stain actin fibers in mammalian cells.
- 3) Fluorescent reported molecules, called fluorophores such as fluorescein, can bind the target of interest through a different chemically-linked molecule.

Immunofluorescence is a widely used method of labeling the molecule of interest. It is an antibody-based technique that uses the highly specific binding of an antibody to its antigen in order to label specific proteins or other molecules within the cell. A sample is treated with a primary antibody, specific to the molecule of interest. A fluorophore can be directly conjugated to the primary antibody. Alternatively a secondary antibody, conjugated to a fluorophore, which binds specifically to the first antibody, can be used.

12.4.3.2. *Biological Samples Expressing a Fluorescent Protein*

The modern understanding of genetics and the techniques available for modifying DNA allow scientists to genetically modify proteins so as to carry a fluorescent protein reporter. In biological samples, this allows a scientist to make a protein, directly of interest, fluorescent.

12.4.3.3. *Autofluorescence*

Autofluorescence is the natural fluorescence of a substance. Many fluorescent substances occur in nature, and they are widely distributed in plants, animals, and minerals. In the human

body, bone has a blue autofluorescence. [27] Human teeth fluoresce blue in UV light, and dentine fluoresces more strongly than enamel. The fluorescence of carious dentine and enamel is weaker than that of normal material in the same tooth. [28]

12.4.4. Protection against Photobleaching

Photobleaching fluorophores will decrease the fluorescence signal and severely limit the observation time by fluorescent microscopy. Photodynamic photobleaching is the most common type of photobleaching, involving the interaction of the fluorophore with light and oxygen. Minimizing illumination can protect against photobleaching, which, however, will also reduce the measurable signal. Another solution is to apply photoprotective scavenger chemicals, such as n-propylgallate, to specimens. Singlet oxygen quenchers, such as histidine, diphenyl-isobenzofuran, or crocetin (a water-soluble carotenoid), can also be employed. The use of a computer-controlled electronic shutter that is open only when experimental data is being collected will prolong filter life as well as keep sample exposure to harmful radiation at a minimum.

12.4.5. Optimizing Image Brightness and Resolution

Optimizing image brightness can be performed by carefully adjusting fluorescent microscopy in the following aspects:

- 1) The sample should be supplied with sufficient light energy for excitation at the appropriate wavelength.
- 2) The emitted fluorescence should be observed without any contamination from the excitation light.
- 3) The light source must provide a large amount of excitation energy in a very narrow range of the spectrum.
- 4) Appropriate filters that transmit the required wavelengths but block the rest should be used.
- 5) The objectives should have a high transmission from the UV light through to a large part of the visible spectrum.
- 6) Objectives with the highest NA should be employed. A good rule of thumb is that if the objective aperture is doubled in size, approximately four times more fluorescence light can be gathered.
- 7) Non-autofluorescent, PCB-free immersion oil with the proper refractive index ($n=1.51$), should be used to eliminate loss of light caused by reflection on surfaces.
- 8) None of the optical components of the microscope should be autofluorescent.
- 9) The objective lenses must be clean.
- 10) Coverslips of the appropriate thickness should be used.
- 11) For objectives with $NA > 0.7$ the thickness of the cover slip can vary ± 0.01 mm (from 0.17 mm) and still provide a high-resolution image.
- 12) For objectives with NA between 0.3 and 0.7 the cover slip thickness can vary by ± 0.03 mm and still provide high-resolution images.

- 13) Air bubbles must be avoided in the immersion-oil layer.
- 14) To improve the contrast, the field diaphragm should be closed down.

12.4.6. Observation of Fluorochrome-Labeled Bone with Fluorescence Microscopy

The use of fluorochromes in bone research is a widely accepted technique for the study of bone formation and bone remodeling dynamics. Many studies also adopt this technique to see the regeneration and remodeling of bone surrounding implants. [29,30].

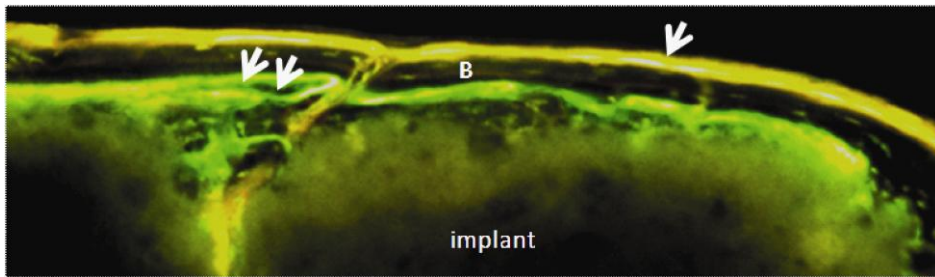


Image courtesy of Dr. A.L.J.J. (Ton) Bronckers from Academic Center for Dentistry Amsterdam, VU University and University of Amsterdam.

Figure 12.9. Fluorescence micrograph of bone formation surrounding an implant. Bone is labeled with calcein (green, double arrow), and subsequently with tetracyclin (yellow, single arrow). The tissue was embedded in methyl methacrylate.

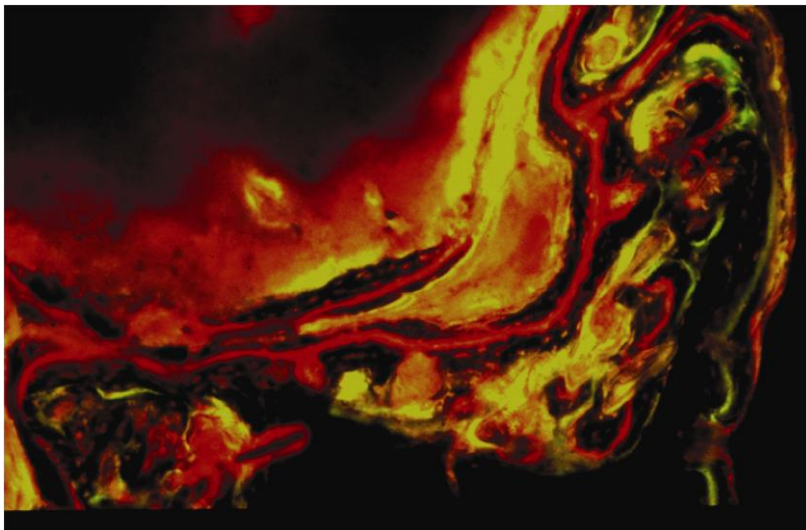


Image courtesy of Dr. A.L.J.J. (Ton) Bronckers from Academic Center for Dentistry Amsterdam, VU University and University of Amsterdam.

Figure 12.10. Fluorescence micrograph of bone formation surrounding an implant. Bone is labeled calcein (green), tetracyclin (yellow) and alizarin red (red). The tissue was embedded in methyl methacrylate.

Using fluorochrome in animal models makes it possible to determine the onset time and location of osteogenesis, which are the fundamental parameters in bone tissue engineering studies. Many types of fluorochromes exist. It is complicated and can be confusing for selecting the appropriate type, the appropriate concentration, the route of administration, and methods of visualization. They can all potentially affect the outcome of fluorescence microscopy.

When bounding to calcium ions, fluorochrome labels can be incorporated at sites of mineralization in the form of hydroxyapatite crystals. The unincorporated label is rapidly excreted by the kidneys, resulting in a peak urine concentration. The fluorescent label demarcates the mineralization front at the time of administration and can be detected in histological sections without any further staining or decalcification. By administering different labels at specific time intervals, bone formation can be followed in time (Figure 12–9 and 12–10). Recently, van Gaalen et al. gave a thorough review on the use of fluorochrome in bone tissue engineering. [31].

12.5. Quantitative Microscopy

Quantitative analysis is indispensable for precisely analyzing the biological response of host tissues and performance of implanted biomaterials. In the field of dental implant research, the quantitative approach is classically applied to estimate various tissue components such as bone-implant contact (BIC), [32] bone fractions and degradation of biomaterials. [33] The unbiased estimation of three-dimensional (3D) structures can be obtained from two-dimensional (2D) histological sections through the science known as stereology. [34] Stereology provides practical techniques for extracting quantitative information about a three-dimensional material from measurements made on two-dimensional planar sections of the material. The stereological approach has a statistical basis in that it is founded on calculations relating to geometrical probabilities. In this section, we describe a few of the basic principles that must be respected for an unbiased estimation of 3D biological structures. Emphasis will be placed on the practical methods that are currently employed to estimate the more commonly determined parameters, such as volume fraction and bone implant contact.

12.5.1. Sampling Strategy

As opposed to other histological and pathological analysis, the research in the field of dental implantology bears certain special characteristics: it is commonly pursued by researchers to show the cross-section that is parallel to the longitudinal axis of an implant and is through the geometrical center of the implant. Moreover, only one or two sections could be obtained from one implant. Therefore, the sampling strategy is largely influenced by the position of the implants and the area of interest in the surrounding tissues.

There are two basic sampling strategies in the field of dental implantology:

- 1) Select a special 2-dimensional section and compare the data from the 2-dimensional section among different groups;
- 2) Use 2-dimensional sections to extrapolate 3-dimensional structures and compare the estimated 3-dimensional data among different groups.

In the field of implantology research, the first strategy is usually adopted to select the sections for bone-implant contacts and bone fractions, especially for the implants that are implanted in bone with a one-side (e.g. lingual or labial/buccal side) bone defect. Usually, the cross-section is done vertically to the mesio-distal bone wall and/or in the middle of the bone defect. This section is not highly representative of the tissue response over-all on the surface of the implant within the one-side bone defect, but is a specially defined section for a group. In a well-controlled experiment, if the sample size is sufficient, the sections derived from this kind of sampling method can be used to compare the different tissue response among different groups. However, the quantitative data obtained from these sections should not be extrapolated to estimate the overall tissue response in area of interest.

The second strategy can be used to estimate 3-dimensional structures from 2-dimensional sections. This science is named stereology. Stereology is a method that utilizes random, systematic sampling to provide unbiased and quantitative data. In order to extrapolate from a few plane sections to the three-dimensional material, essentially the sections must be “typical” or “representative” of the entire material. There are basically two approaches to ensure this:

- 1) Model-based sampling inference: we assume that any plane section is typical (e.g. assume that the material is completely homogeneous);
- 2) Design-based sampling inference [35]: we select plane sections at random, according to a specified random sampling.

The first approach is the one that was used in classical stereology. Extrapolation from the sample to the 3-D material depends on the assumption that the material is homogeneous. This effectively postulates a statistical model of the material. This approach can be used in both non-decalcified and decalcified tissue. Data is normally obtained from one or several sections without following a systematic and random protocol, which can be biased.

The second approach is the one typically used in modern stereology. Instead of relying on model assumptions about the three-dimensional material, we take our sample of plane sections by following a randomized sampling design; for example, choosing a random position at which to start cutting the material. Extrapolation from the sample to the 3-D material is valid because of the randomness of the sampling design, so this is called a *design-based* sampling inference. Design-based stereological methods can be applied to materials which are inhomogeneous or cannot be assumed to be homogeneous. These methods have gained increasing popularity in the biomedical sciences, especially in lung-, kidney-, bone-, cancer- and neuro-science. [9, 36, 37] Many of these applications are directed towards determining the number of elements in a particular structure.

For the research in the field of dental implantology, this method is typically used to evaluate the tissue response surrounding the dental implants that are implanted in bone or bone defects. A well-defined systematic and random selection protocol should be followed to select the cross-sections that are parallel to and through the centers of implants in one group.

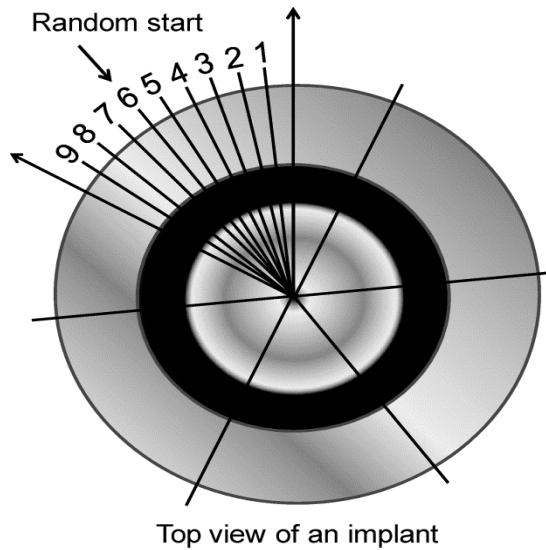


Figure 12.11. Schematic graph depicting a systematic and random sampling protocol to select sectioning-position on the top view of a dental implant.

With a sufficient sample size, the 2-dimensional data could be extrapolated to estimate the 3-dimensional data on the overall surfaces of implants. We take a study with 3 implants per group as an example. Figure 12–11 shows a sampling protocol on the top-view of an implant. The starting position should be set in a uniform direction for all the implants, such as the distal/mesial direction in jaw/femoral bone and forehead in calvarias. The angles between each adjacent potential sectioning position is $180^\circ/3=60^\circ$. The first left-turning 60° angle is divided into 10 parts. And then, according to a random number list, we chose a number; in this case, we took 6. Then, we set the random start for sample at number 1 and then for sample number 2; the sectioning position will set by turning 60° from the random start. The sample number 3 will be sectioned through the plan at the position of 120° away from the random start. Then, the systematic sampling protocol is applied for 3 implants in one group. For another group, we can restart with this sampling process. In this kind of research, sample size should always be checked for its efficiency because only one or two sections can be obtained through this kind of sampling protocol.

Another random start and systematic sampling protocol can be applied to evaluate biomaterials as well as dental implants. Figure 12–12 shows a systematic and random sampling protocol for selecting sectioning positions on the tissue block of an implanted biomaterial. The selection of the direction of section planes is influenced by the geometric size of samples and the intended numbers of sections. We usually use vertical uniform random (or VUR) sections [38] for some stereological methods, particularly with tissues that have well defined orientations, such as skin, muscle, and bone. A VUR section is less random than a completely random section. A common use of VUR sections is estimating the length, surface area and volume fraction. VUR sections are sometimes appropriate and are described below.

A VUR section or slice is made in 3 steps:

- 1) Select an arbitrary vertical axis, or select a horizontal plane and then determine the axis perpendicular to that plane. Either way, an orientation is selected that is called the vertical axis.
- 2) Rotate the material in a random manner about the vertical axis.
- 3) Cut sections or slices parallel to the vertical axis that have a random start position. The distance (d) between adjacent sections is the same. The distance will be used to estimate the 3-dimensional structures through the data obtained from 2-dimensional sections.

The Cavalieri method is used to estimate the total volume of a reference space. [39] The estimator is often used in conjunction with other estimators that estimate density such as number density, length density, or surface density.

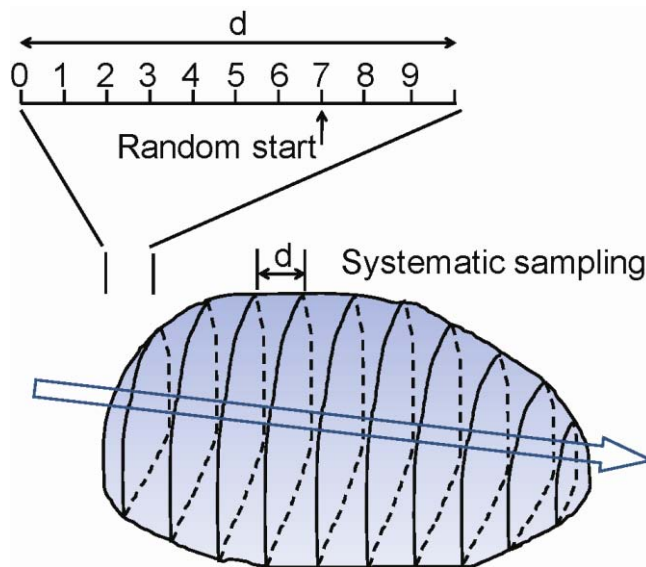


Figure 12.12. A schematic graph depicting a systematic and random sampling protocol to select sectioning-position on the tissue block of an implanted biomaterial.

12.5.2. The Estimation of the Total Volume (Cavalieri's Method) and the Volume Fraction of a Component

The Cavalieri estimator is prone to over projection if the set of slices are thick. This is explained in the section on volume formulas. The sections are sampled using the fractionator principle. A rule of thumb is to use 10 to 15 sections. Suppose that the tissue is sectioned into 100 sections. If every 8th section is used, then around 12 sections are used. This is usually an adequate number of sections. The Cavalieri estimator is usually performed using a point grid. The points need to be appropriately spaced. The points are usually spaced equally both across and down. A rule of thumb is to count 550 to 650 points for one component of interest in each group. If there are 6 samples per group, and 10 sections per sample, this rule is met if an average of 10 counts per section is made per section. Some experimentation is usually required to determine an adequate spacing of the point grid.

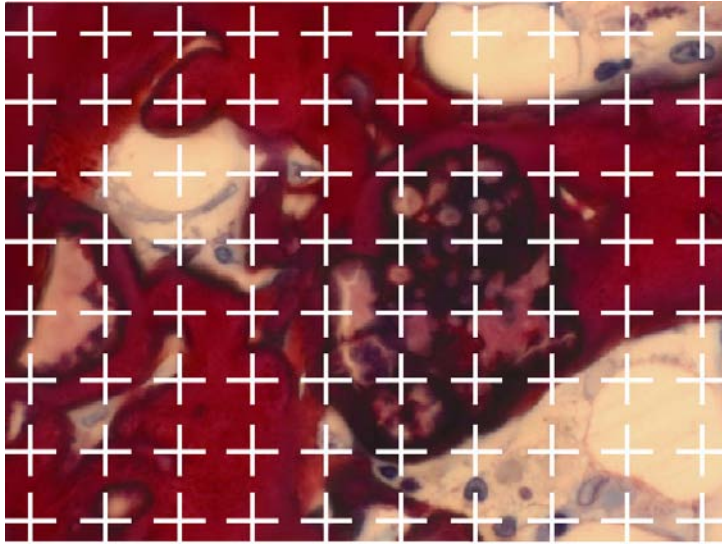


Figure 12.13. Schematic graph depicting the cavalieri method with a point grid overlaid on a tissue section.

Regions that have complicated profiles, or profiles that are thin, require closer points than simple blob shapes. The distance between points can be used to compute the intensity of the probe. The point grid can either be printed on a transparent plastic film, which can be used to measure on printed pictures, or be overlaid on electronic images. Therefore, an area of each grid point (a_p) = (the real distance between adjacent point).²

Toss the point probe randomly onto each section (Figure 12–13). Count how many points hit the region of interest. Keep a record of how many points hit each section. The results are used to compute the total volume and the volume fractions. Following is a sample:

The distance between each adjacent section (d) is 1mm. The a_p for reference space (a_{pr}) = 0.5625mm^2 . The a_p for a component of interest (a_{pc}) = 0.2250mm^2 . The point counts of the reference space from all sections are 108 and the point counts of a component of interest from all sections are 112. Then, the total volume of reference space = $d \times a_{pr} \times \text{counting} = 1\text{mm} \times 0.5625\text{mm}^2 \times 108 = 60.75\text{mm}^3$; the total volume of a component of interest is = $d \times a_{pc} \times \text{counting} = 1\text{mm} \times 0.2250\text{mm}^2 \times 112 = 25.2\text{mm}^3$. The volume fraction of the component of interest within the reference space = $25.2/60.75 = 41.5\%$. The volume fraction of the component of interest within the reference space can also be calculated using formula: $d \times a_{pr} \times \text{counting} / d \times a_{pc} \times \text{counting} = a_{pr} \times \text{counting} / a_{pc} \times \text{counting}$.

12.5.3. Estimation of Surface Area

In addition to the volume density, the surface area of a component is another important parameter for evaluating the 3-dimensional structures of biological tissues such as bone and biomaterials. Before the 1980's, the estimation of the surface density was based on the assumption of either (1) uniform orientation distribution or isotropy of the surface, or (2) isotropically orientated sections. However, it was later shown that bone was anisotropic [40] and those methods might produce biased results. Until 1987, Versterby et al. reported an unbiased estimation method where cycloid grid and VUR section was adopted. [41] Based on

this theory, a vertical spatial grid (VSG) (Figure 12–14) that was developed by Cruz-Orive and Howard, is a test probe comprising of two systems of rolling cycloids. The test system has a known length of cycloid per point (l/p). In order to estimate surface density, two counts need to be made on this image:

1. The number of intersections between the cycloid lines and the boundary of interest (I),
2. The number of points that land within the reference space (P).

If a number of micrographs are used for surface density estimation, a separate count is made for the number of intersections (I_i) and points hitting the reference space (P_i) on each image. Surface density is then estimated from the formula $S_v = 2 \times \sum I_i / [(l/p) \times P_i]$.

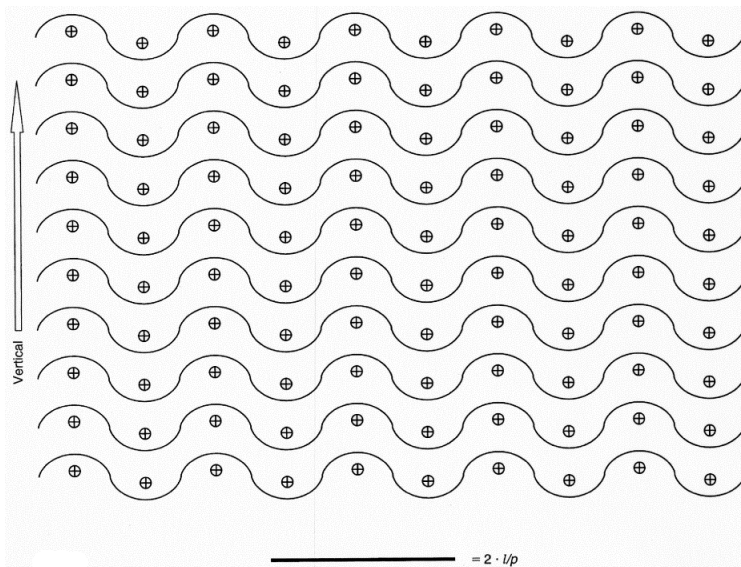


Figure 12.14. A vertical spatial grid for to measuring the surface area density on 2-dimensional sections.

Conclusion

The principle of histological process and data analysis were described briefly; the related protocols were given in detail in tables; and the necessary requirements were introduced in this chapter. It is hoped to be a text book for studying the bone-implant interface. A full understanding of the principle and correct performance for each step will lead to reproducible results in the field of implant research.

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Chapter XIII

Micro-CT Application in Oral Implant Research

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Abstract

The principle of computed tomography (CT) is based on a mathematical conversion of multiple x-ray projection images taken from many angles into a stack of cross-sectional image slices. This stack of images is often referred to as the three-dimensional image. The technique of the computed tomography was pioneered by Hounsfield in 1972. Since then, the medical CT scanners became important diagnostic imaging instruments in the hospitals. Later, specialized high spatial resolution microcomputed tomography (micro-CT) systems were developed with the aim of imaging small objects.

Micro-CT has been used extensively to generate high-resolution three-dimensional images of calcified tissues in different animal models and in humans. Nondestructive three-dimensional (3D) micro-CT imaging requires adequate tools for the numerical assessment of the experimental systems. Such quantitative tools should be user-friendly and intuitive, not too complex for the biomaterials researcher to implement. They should be accurate, repeatable and suitable for laboratory application.

Micro-CT imaging allows for the three-dimensional visualization and quantification of peri-implant bone and is, therefore, a powerful tool for understanding biological reactions around implants. The image data can also be used to perform the biomechanical simulation studies of implants in bones by using the finite element approach. Recent advances in the development of *in vivo* micro-CT scanners give the possibility to follow-up the same experimental animal at different time points. Therefore, the animal experiments can be refined and the number of animals reduced. Still, due to the imaging

artifacts and the insufficient resolution of present micro-CT scanners, an accurate assessment of the direct interfacial contact area between the implant and peri-implant bone is challenging. Histological sectioning still provides superior resolution, and the micro-CT studies should be supplemented by using microscopy techniques.

This chapter traces the history and development of micro-CT. This chapter will also review the current application of micro-CT in different areas of bone and biomaterials research. Finally, details of studies in the area of implant research are highlighted along with the advantages and disadvantage of micro-CT that have been discussed in this chapter.

13.1. Introduction

Computed tomography (CT) is an imaging technique that uses x-rays to obtain the cross-sectional reconstructions of three-dimensional objects. This imaging technique was introduced by Geoffrey Hounsfield in 1972 [1], using the principles developed by Radon in the early 20th century. Medical CT scanners became important diagnostic imaging instruments in hospitals. Computed tomography offered unique possibilities of a non-destructive examination of the internal microstructure of thick objects, even in liquid or gaseous environments. Therefore, the technique was of great potential for applications in different fields of scientific research. [2] However, in the 1980's, the resolution of medical CT scanners was around 1 mm. [3] The need for high resolution microscopes, based on the principles of computed tomography, triggered the development of the purposely built high resolution CT systems, which became known as micro-computed tomography (micro-CT) systems. Regardless of the beam type (e.g. fan, cone or spiral), type of x-ray source and detector, CT systems with the voxel resolution between 1 and 100 μm are generally referred to as micro-CT. [4] However, it should be noted that the resolutions of modern dental [5] and pQCT scanners [6] are approaching or already in this range. [4].

In 1989, Feldkamp and colleagues reported the development of a high-resolution CT system [7] based on the previously published cone-beam reconstruction algorithm. [8] This device was originally designed to image small structural defects in ceramic materials but was shown to be useful for the imaging and characterization of cancellous bone specimens. The voxel resolution of the reconstructed object was 40 μm . Soon, micro-CT systems were developed by others [9-11, 38] with the aim of imaging small objects.

In a typical micro-CT device, a low-energy (10-100 keV) conical beam from a micro-focus X-ray tube passes through the investigated object (Figure 13–1A). The attenuated radiation is recorded by a planar X-ray sensitive detector (CCD camera) as an enlarged radiographic projection of the object. The magnification of this radiographic projection is determined by the ratio between the X-ray tube-to-object and object-to-detector distances [3]. Unlike medical scanners, the positions of the X-ray tube and detector in micro-CT devices are fixed in place, and it is the investigated object which is being rotated at increment angles through an arc of e.g. 180 or 360 degrees. Based on the angular radiographic projections acquired while the object rotates, a computer performs the direct reconstruction of two-dimensional projection data into a three-dimensional array of points (Figure 13–1B). Most of the micro-CT systems are designed to perform the imaging of *ex vivo* samples. However, *in vivo* micro-CT scanners, for the imaging of small laboratory animals, are also available.

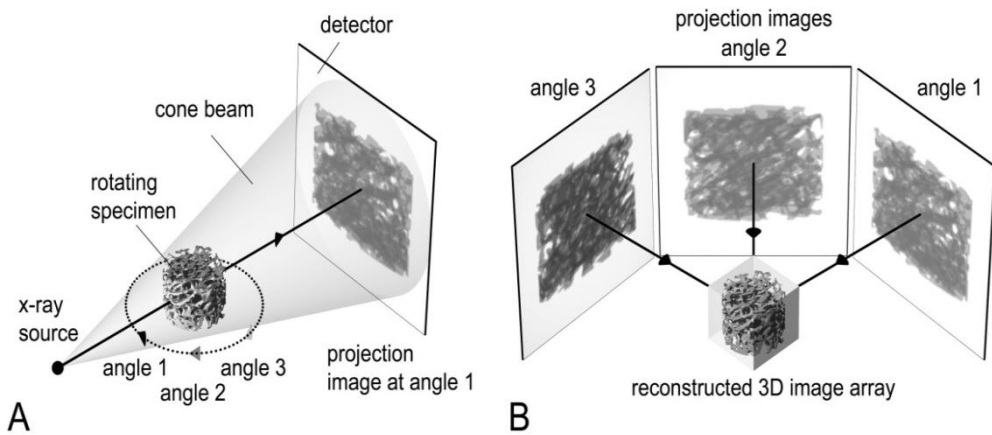


Figure 13.1. Principle of operation of a typical micro-CT system: A) A micro-focus x-ray source illuminates the rotating sample with a cone beam. A planar x-ray detector collects magnified projection images; B) Based on a back projection algorithm, the 3D image array is synthesized from the projection images.

Synchrotron X-ray tomography systems (SR micro-CT) utilize monochromatic synchrotron radiation sources. The reconstruction procedure is simple due to the parallelism of the x-ray beam. The spatial resolution of SR micro-CT could be adjusted down to 1 μm . [12] The applications of these systems, however, are limited by the availability of synchrotron facilities.

Recently, nano-CT systems have been introduced. The resolution of these systems is in the sub-micron range values of 150 nm [13], and even 50 nm² were reported. These commercial systems become essential components of many academic and industrial research laboratories. A wide range of specimens could be directly examined by using Micro-CT, including mineralized tissues such as teeth; bone; and materials such as ceramics, polymers, tissue-engineering scaffolds. Micro-CT imaging could also be extended to soft tissues, such as lungs that have been infiltrated or perfused with a contrast agent, having a higher density than the surrounding tissue.

13.2. Micro-Computed Tomography in the Three-Dimensional Analysis of Bone

Micro-CT became a widely used tool in biomedical research: it has been used for the evaluation of the three-dimensional architecture of trabecular bone [7, 11, 14], synthetic porous scaffold materials, [15] as well as bone ingrowth into these porous structures. [16] A number of studies have also been concerned with finite element (FE) modeling [17] and the evaluation of bone mineral density. [18,19] In clinical studies, micro-CT imaging can demonstrate tremendous differences in the three-dimensional trabecular bone structure for patients with normal and poor bone quality [20] (Figure 13–2).

The following example demonstrates the popularity of the micro-CT: at the time of writing this chapter, a search with keywords “bone” and “micro-CT”, limited to the last 10

years, returned 1,283 results in the PubMed database. In this chapter, no attempt was made to review these results.

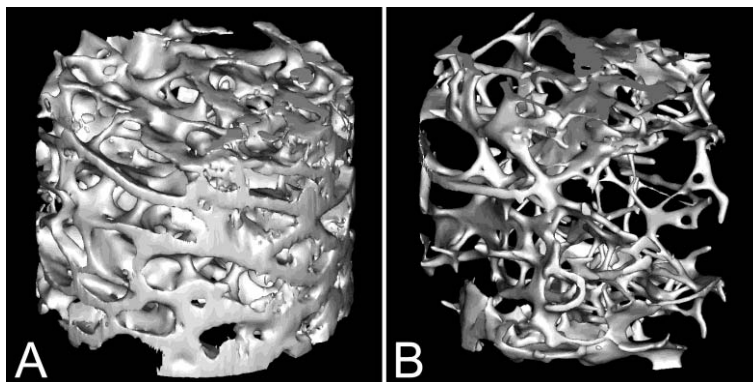


Figure 13.2. Three-dimensional trabecular bone architecture of A) normal and B) osteoporotic intertrochanteric cancellous bone specimens from human patients.

We only present the basic information on micro-CT imaging techniques. A comprehensive review is given in a recent paper by Buxsein and colleagues. [21] This article also contains guidelines for the reporting of the results from micro-CT-based studies.

Directly from the introduction of the micro-CT technology, the non-destructive three-dimensional characterization of cancellous bone became the focal point of many studies. [7, 14] Parameters used in two-dimensional histomorphometry [22] were adapted for the analysis of the three-dimensional micro-CT data sets. In addition, new methods for analysis of the three-dimensional structures were introduced. [23–26] Good correlations with histomorphometric analysis were reported. [14, 27] The selection of segmentation procedures (thresholding) was reported as being essential for the successful analysis of bone structures. [14, 28] The most common parameters for the characterization of three-dimensional structures are given in Table 13–1 with brief explanations.

Studies of the cortical bone gained less attention. These studies were mainly concerned with cortical bone geometry, density and strength. [29, 30] However, SR micro-CT was applied for the analysis of bone porosity in small animals. [31, 32] With the improvements in the resolution of the micro-CT scanners, the three-dimensional structure of the human cortical bone at the midshaft of the femur could be characterized by micro-CT [33, 34]. Structural parameters analogous to the ones used in cancellous bone were suggested: [35] percent porosity or “canal volume fraction” (CaV/TV), canal surface to volume ratio (CaS/CaV), mean canal diameter (CaDm), mean canal separation (CaSp), canal number (CaN), degree of anisotropy (DA) and canal connectivity density (CaConnD), a measure of the number of interconnections or branching points in the canal network. Figure 13–3 demonstrates the possibilities of micro-CT imaging in studies of the cancellous and cortical bone.

Different techniques have been developed for non-invasive measurements of bone mineral density (BMD) at different skeletal sites. [36] In clinical use, these characterization techniques are either two-dimensional (X-ray absorptiometry, DXA) or the resolution is not enough to study individual trabeculae (QCT and pQCT). Micro-CT allows measuring of the tissue mineral density (TMD). [37, 21] Unlike BMD, which includes bone and bone marrow attenuation values, TMD is calculated from the attenuation of only the bone tissue. Attenuation values in micro-CT data are converted to physical density (mg/cm^3 of

hydroxyapatite, HA). Early attempts to use micro-CT for measuring the mineral content in teeth and bone were made by Elliot. [38, 39].

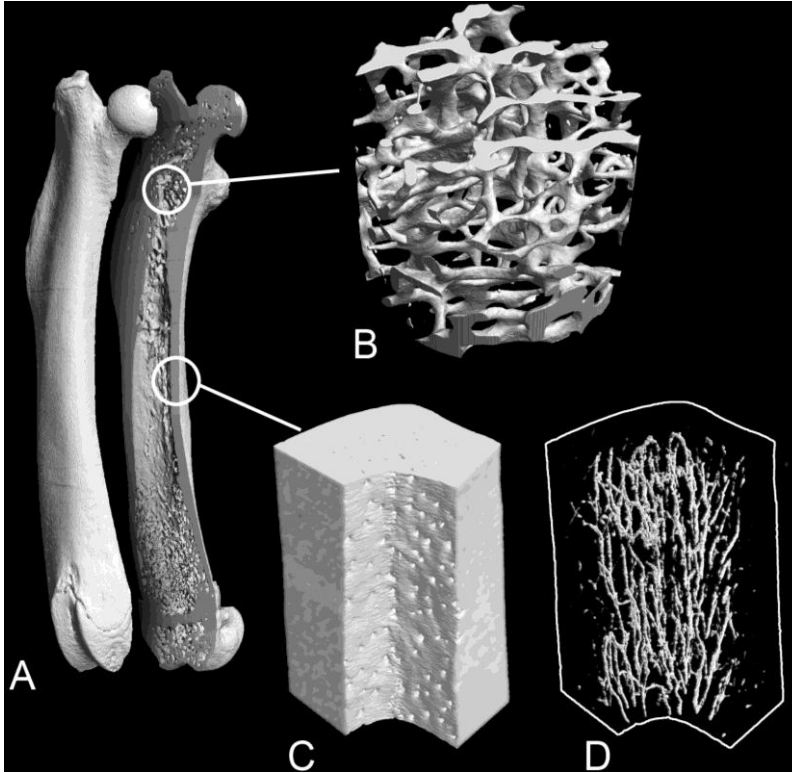


Figure 13.3. Imaging of bone with micro-CT: A) Whole rat femur; B) A sample of trabecular bone; C) a sample of cortical bone; D) The network of channels in the cortical bone sample is visualized.

TMD measurements are challenging, and there are three main sources of errors: partial volume effect, beam hardening and the thickness of the surrounding material. [40] The partial volume effect can be corrected by complex methods, e.g. calibration with aluminum foils of different thickness, but an easier solution is scanning with a high enough resolution [40]. Beam-hardening, due to the broad spectra of energies in micro-CT, manifests in the increased density of the voxels adjunct to the surface of the scanned object. Beam-hardening correction algorithms have been designed to compensate for this effect. Proper selection of imaging filters and X-ray voltage also helps to minimize beam-hardening. [40, 41] The thickness of surrounding tissues has an effect on the density in the micro-CT image. The best results can be obtained by the ex-vivo scanning of bone samples without any surrounding tissues. [40] Like in any quantitative CT imaging, calibration with phantom materials is essential. [18, 40, 42-44].

SR micro-CT is a method of choice to perform quantitative densitometry measurements. [18, 45] SR micro-CT allows high image resolution and is devoid of beam-hardening artifacts due to the use of a monoenergetic synchrotron beam. [45] The main restriction of this method is the limited access to the synchrotron facilities. However, it has been demonstrated that micro-CT gives comparable quantitative densitometry results. The minor differences between micro-CT and SR micro-CT results were attributed to the physics of image formation and to the imaging conditions. [18].

Table 13.1. Typical morphometric parameters used in micro-CT-based 3D analysis [40, 22, 23]

Parameter	Abbreviation		Unit	Description
	General	Bone		
Percent object volume	Obj.V/TV	BV/TV	%	Ratio of volume of binarised objects (Obj.V or bone volume, BV) inside a VOI to total volume (TV) of VOI expressed as percentage.
Object surface to volume ratio	Obj.S/Obj.V	BS/BV	mm ⁻¹	Ratio of surface area of binarised objects (Obj.S or bone surface, BS) to volume of these objects. Useful in characterization of thickness and complexity of a 3D structure.
Object surface to volume ratio	Obj.S/TV	BS/TV	mm ⁻¹	Ratio of surface area of binarised objects (Obj.S or bone surface, BS) to total volume (TV) of VOI.
Intersection surface	i.S	i.S	mm ²	Area of the fraction of surface of VOI intersected by binarised objects. Useful in characterization of e.g. bone formation at a predefined distance from an implant.
Fragmentation index and Trabecular bone pattern factor	Fr.I	Tb.Pf	mm ⁻¹	An inverse index of connectivity. Based on the principle that concavity of a structure indicates connectivity while convexity indicates disconnected structures. This relative index is useful for comparison of connectivity of different structures in 3D space. Absolute values are of little meaning.
Euler-Poincare number	Eu.N (Conn.D)	Eu.N (Conn.D)	mm ⁻¹	An index of connectivity. It indicates the degree of multiple connections in a 3D structure.
Structure model index	SMI	SMI	n/a	SMI indicates the relative prevalence of rods and plates in a 3D structure. Based on surface convexity measurement. This index is useful for evaluation of effect of osteoporosis on a trabecular bone structure which is signified by transition from plate-like structure (SMI = 0) to rod-like structure (SMI=3). For an ideal structure comprised of spheres SMI =4.
Structure thickness and Trabecular thickness	St.Th	Tb.Th	mm	Local thickness in a 3D-structure, e.g. thickness of an individual trabecula in a trabecular bone. This index is typically reported as a mean value or as a histogram of local thicknesses.
Structure linear density and Trabecular number	St.Li.Dn	Tb.N	mm ⁻¹	The index is calculated as the number of traversals across a trabecular 3D structure per unit length on a random linear path through the VOI.
Structure separation and Trabecular separation	Sr.Sp	Tb.Sp	mm	Local thickness of voids in a 3D-structure, e.g. thickness of an individual pore in a porous structure. This index is typically reported as a mean value or as a histogram of local thicknesses.
Degree of anisotropy	DA	DA	n/a	The measure of preferential alignment of structures along a directional axis. This index is determined by mean intercept length (MIL) analysis method. DA is the ratio of the maximum eigenvalue to the minimum

Parameter	Abbreviation		Unit	Description
	General	Bone		
				eigenvalue in MIL analysis. For convenience, the formula is often modified to give 0 for total isotropy and 1 for total anisotropy.
Eigenvalue	Eigenvalue	Eigenvalue	n/a	In MIL analysis, each of the three eigenvalues is an index of the relative MIL values in each of the three axes described by the eigenvectors.
Fractal dimension	FD	FD	n/a	Fractal dimension is a measure of surface complexity and self-similarity of a 3D-structure. It is calculated using e.g. Kolmogorov method.
Porosity	Po	Po	%	Total porosity is the volume of all voids in binarised objects to total volume of VOI expressed as percentage. Open porosity is the volume of voids connected to the surface of the binarised objects to total volume of VOI expressed as percentage. Closed porosity is the volume of voids not connected to the surface of the binarised objects to total volume of VOI expressed as percentage.

Gravimetric methods, which estimate the ash mass and density in bone samples, were applied to verify the TMD measurements performed by micro-CT [41-43, 46] and SR micro-CT. [46] In general, good correlations were demonstrated between micro-CT densitometry and gravimetric methods. Despite good correlations, there were notable differences in the results between micro-CT and SR micro-CT, [46] suggesting that micro-CT results were underestimated.

However, micro-CT-based densitometry results should be interpreted with caution. In a recent study, [20] performed in a selected group of female total hip arthroplasty patients, intertrochanteric cancellous bone biopsies were taken from the site of the stem implantation. TMD measurements were done by micro-CT on these bone biopsies. All patients underwent DXA measurements. The systemic BMD level, as well as the local DXA values of the trochanteric region (BMD and T-score value), showed a significant association with many of the microstructural micro-CT parameters of the cancellous bone. However, no associations between DXA-measurements and TMD measurements were detected. This result may be due to limitations and artifacts in the micro-CT-based measurements of TMD. [37, 43].

13.3. Micro-Computed Tomography in the Three-Dimensional Analysis of Porous Biomaterials

A large number of bone graft substitute materials are being marketed as porous. However, the reported porosity is usually just the ratio of the total pore volume to the apparent volume of the sample [47]. This simple definition of porosity does not adequately describe the properties of porous bone graft substitute materials. Consequently, the comparison of porous biomaterials from different manufacturers is not plausible. However, the significance of architectural features for the performance of biomaterials in a physiological environment is well understood. [48-50].

Traditional methods for analyzing porosity are based on microscopy, pycnometry, gas absorption, etc. [47] Mercury intrusion porosimetry is a typical method for analyzing porosity in materials. [47, 51] However, these methods have intrinsic limitations in respect to the analysis of material structure and morphology. [15, 49, 50] Micro-CT provides a non-destructive, quantitative assessment of porosity and the interconnectivity of porous microstructures; [15, 16, 49, 50, 52-59] furthermore, the correlations of micro-CT based measurements with the *in vivo* performance of porous biomaterials were demonstrated. [49, 56] In addition, it seems feasible that a standard approach to the characterization of porous biomaterials may be developed, for example, based on the method described by Moore and colleagues. [50].

13.4. Micro-Computed Tomography in Oral Implants Research

Oral and orthopaedic implants are often made of titanium and its alloys. Machined titanium surfaces provide good bone bonding in healthy bone. However, in poor bone conditions, osteoconductive surface treatments or the biofunctionalization of the implant surface may provide more predictable osseointegration. Most oral implants have specific surface structures which are claimed to enhance primary stability of an implant and the bone healing process.

In biomaterials research, the analysis of the interface between the synthetic implant material and host tissues is of primary importance. Since the mechanical behavior of bone is a critical factor in the attainment and maintenance of osseointegration, several classification systems and procedures were advocated for assessing bone quality. The osseointegration of the interface has been commonly evaluated by histomorphometric methods. However, histomorphometry is a destructive method, and the same specimens can not be characterized by other methods, e.g. biomechanically. Another disadvantage of the histomorphometric method is that a two-dimensional slice represents only a part of the entire interface. In order to obtain more comprehensive information, a series of parallel cross-sectional slices can be prepared. However, the physical preparation of such a series of sections could be time-consuming and the result could be dependent on section preparation techniques. Thus, the use of non-destructive, three-dimensional imaging techniques is justified for analyzing biomaterial-host tissue interfaces. Recently, micro-CT has been applied in the analysis of the peri-implant bone around the titanium implants with the characterization of the trabecular bone being of primary interest. Micro-CT imaging provides a spatial representation of bone formations at the implant surface and the peri-implant region. Further analysis of the data enables a qualitative and quantitative assessment for the integration of oral implants with bone.

While micro-CT provides the possibility of non-destructive, fast and three-dimensional imaging, it has certain fundamental limitations to the imaging of metallic implants in bone. Metallic implants are known to produce artifacts in medical CT scanners, and the same applies for micro-CT imaging. In x-ray imaging, the attenuation length of the x-ray is inversely proportional to the mass density and atomic number of the material. As a result, higher energies are needed to study thick and heavy materials whereas lighter materials

require lower x-ray energies. Therefore, the imaging of samples containing both light (e.g. bone) and heavy (e.g. metallic implant) materials is technically challenging as the attenuation may differ by a factor of one hundred [3]. The energy of the x-ray tube should be tuned to allow for the imaging of both types of materials. There is a risk that a lighter object will either disappear or be obscured by the heavier in some of the projections, rendering an artifact-free reconstruction impossible. Figure 13–4 demonstrates a micro-CT imaging of oral titanium implants (ITI[®], Straumann AG, Switzerland) in a dog mandible.

In micro CT imaging, beam-hardening, partial volume effect, scatter and noise make it difficult to distinguish the interface between the implant and the bone. [60] Therefore, with some exceptions, [61-63] efforts in analyzing the peri-implant bone are generally concentrated on the characterization of the bone volume in proximity to the bone-implant interface. [64] The successful application of micro-CT in characterizing samples containing bone and implants started around the year 2000. [65-67] These early studies were mainly visualizations of the implants and peri-implant cancellous bone.

There is some inconsistency in the implant research by micro-CT: different commercially available micro-CT systems are being used; experimental setup, animal models and implant design vary from one research group to another. Moreover, there seem to be no synoptic approach to analyzing, interpreting and reporting the results. With the exception of one study, [68] the imaging was performed on *ex vivo* samples.

Ban and colleagues [69] used micro-CT to study a new bone formation around four types of cylindrical implants, with and without electrochemically deposited apatite coatings. The implants were inserted in the cortical bone in the diaphysis of the rabbit femur. Good correlations were observed between the amount of new bone and the bonding strength, measured by a pull-out test. Yip and colleagues [70] studied the osseointegration of oral implants in the cortical bone in the diaphysis of the dog femur. Good correlations were found between two-dimensional histological and micro-CT images; however, no quantification of the peri-implant bone was attempted. Stoppie and colleagues [60] used screw-shaped titanium implants inserted in the femoral condyle of goats to validate the micro-CT technique against the histological serial sectioning (gold standard).

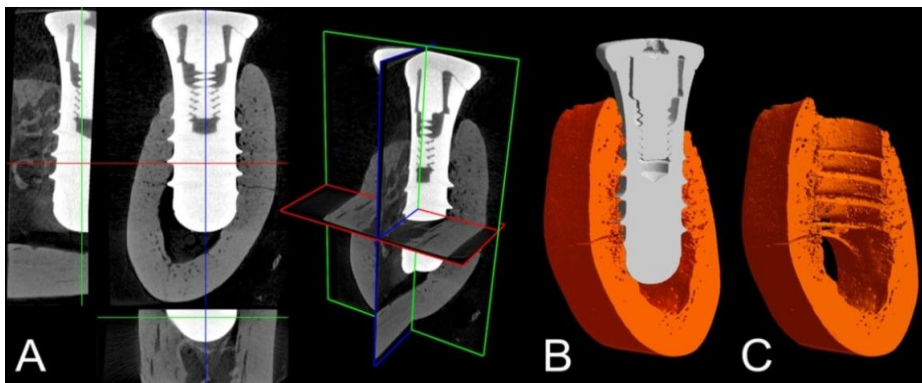


Figure 13.4. Micro-CT Imaging of titanium oral implants (ITI[®] Straumann AG, Switzerland) in dog mandible. Blocks from study by Rossi and colleagues [89] were used to demonstrate the imaging technique: A) Different image planes in the 3D-dataset. Imaging artifacts are clearly visible; B) 3D surface model of the implant in bone; C) Implant removed by segmentation; peri-implant bone is visualized.

Trabecular bone was assessed inside a ring-shaped, apparently 2 mm wide region of interest (ROI) around the implants. Micro-CT was concluded to be a highly reliable tool for the characterization of the trabecular bone; however, bone-implant contact was not possible to measure. De Smet and colleagues [71] studied the effect of the early loading of the screw-shaped implants on the properties of the peri-implant bone. The implants were placed in the diaphyseal area in the tibia of guinea pigs with bicortical implantation. Micro-CT was used to study bone formation inside the 0-500, 500-1000 and 1000-1500 μm zones away from the implant surface. Micro-CT analysis was able to detect significant differences in bone mass evolution among the different implant groups. Stoppie and colleagues [68] performed a validation of an *in vivo* micro-CT system. In a rabbit model, screw-type implants were placed in the trabecular bone of the condyle of the tibia. Micro-CT was used to study bone formation inside the 0-500, 500-1000 and 1000-1500 μm zones away from the implant surface. Low agreement was found between the histological examination and micro-CT. Shouten and colleagues [64] used a similar analysis technique to study peri-implant bone response to two types of oral implants (non-coated, CaP-coated, CaP-coated +TGF-beta1). Implants were placed in the femoral condyles of goats. The superiority of conventional histology over micro-CT was reported. In the zone 0-500 μm , micro-CT data was unreliable.

Nakada and colleagues [19] used micro-CT to study new bone formation and TMD around cylindrical titanium implants with different surface treatments. Implants were placed in the metaphysical area of the proximal femur in rabbits. Analysis was performed inside a standard tube-shaped volume of interest (VOI) around the implants. The analysis revealed differences in BV and TMD for different surface treatments.

Shalabi and colleagues [72] have suggested the use of thin Ti-coated PMMA implants placed in the tibia of goats as an alternative option for analyzing bone contact using micro-CT imaging. Apparently, this was an attempt to improve micro-CT imaging conditions. They concluded that the additional use of microscopical techniques was still required for a proper analysis of the bone-implant interface.

The studies in rats were less diverse. Peter and colleagues [73] studied cylindrical titanium implants coated with HA and loaded with Zoledronate. The implants were inserted in the condyle of osteoporotic rats. Half of the implants were studied by histology and SEM, the other half by micro-CT and pull-out. In the micro-CT analysis, the trabecular bone fraction was studied inside a 500 μm -wide tube-shaped VOI around the implants. Differences in BV/TV demonstrated Zoledronate's effect. Gabet and colleagues [61] studied the effects of parathyroid hormone 1-34 on the anchorage of the screw-shaped implants in a cancellous bone in the rat. Implants were inserted in the proximal metaphysis in the tibia. Trabecular bone was characterized within a VOI, 900 μm away from the implant.

The typical microstructural parameters that can be measured are: bone volume/total volume (BV/TV), bone surface (BS), trabecular thickness (Tb, Th), trabecular separation (Tb, Sp) and bone connectivity (Conn. D). Bone-implant contact was also measured. Highly significant correlations were obtained between the morphometric and biomechanical parameters. Li and colleagues [62] studied the effect of a strontium ranelate treatment on the osseointegration of the HA-coated titanium screws in ovariectomized rats. Implants were placed in the proximal tibia. Trabecular bone was studied inside a 500 μm -wide tube-shaped VOI around the implants. BV/TV, Tb.Th, Tb.TN and Conn.D were measured as well as bone-implant contact. The implants were also subjected to a push out-test. Micro-CT parameters were significantly correlated with biomechanical parameters. The strongest relationship was

between BV/TV and the ultimate push-out force. The effect of the strontium ranelate treatment was confirmed. Maimoun and colleagues [63] performed a study with a similar experimental setup. Implants were placed in the proximal tibia of a rat. They used cylindrical titanium implants with a sandblasted and acid-treated surface. By micro-CT, they studied the same parameters plus SMI. The implants were also subjected to a pull out-test and nano-indentation of peri-implant bone. The effect of the strontium ranelate treatment was confirmed by micro-CT imaging, pull-out and nano-indentation.

Akca and colleagues [74] studied oral implants placed in anterior and posterior regions of a completely edentulous maxilla and mandible of a human cadaver. Micro-CT was employed to study trabecular bone in nine concentric tube-like VOI with a 40 μm width increment. BV/TV, Conn.D, Tb.N, TbH and TbSp. were studied. Correlations were detected between micro-CT-based parameters and insertion torque values.

Recently, micro-CT was also employed to study crestal bone loss around oral implants in a dog model. Park and colleagues [75] performed a pilot study in a dog model to evaluate the effect of the microthread geometry of an oral implant of a scalloped design on the bone resorption in the immediate loaded condition. Yoo and his group studied the influence of premature exposure of the submerged oral implants on early crestal bone loss. Micro-CT was performed on the implantation site to measure the bone height in the peri-implant bone [76].

SR micro-CT is a technique that avoids some of the artifacts present in imaging by conventional micro-CT. Comparisons between SR micro-CT and micro-CT imaging were made using cuboid-shaped titanium alloy implants (with different image modifications) placed in the lower jaw of beagle dogs. [77] The implants were retrieved and imaged using one SR micro-CT and three different micro-CT systems. Sections from all micro-CT systems (bone %) were compared with a classical histology done by serial sectioning. The superiority of SR micro-CT over micro-CT was demonstrated. [77] In further studies, Bernhardt and colleagues [78, 79] studied different biofunctionalized titanium implants. In their studies cylindrical implants with two identical tube-like cavities, designed to compare bone formed inside these cavities with the intact cancellous bone in a tube-like VOI around the cavities. Implants were placed in the femoral condyles in goats. After retrieval, the blocks with bone and implants were imbedded in plastic and studied by SR micro-CT and histology.

A comparison with histology at an early time-point demonstrated the weakness of SR micro-CT in detecting incompletely mineralized bone. For that reason, they recommended not to embed blocks in plastic before imaging with SR micro-CT.

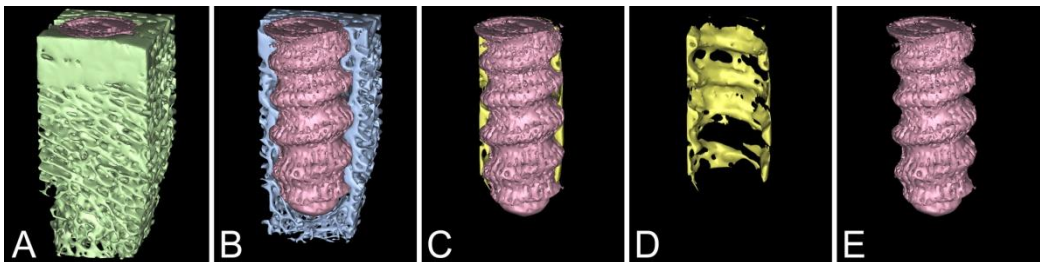


Figure 13.5. Imaging of FRC implants in the pig bone. A) Sample as retrieved; B) Visualization of the FRC screw in trabecular bone (part of the bone is not shown); C) New bone formation within the treads of the FRC implant (part of the bone is not shown); D) New bone formation within the treads of the FRC implant; implant removed by segmentation (part of the bone is not shown); E) FRC implant.

A further study [79] was done with a similar experimental setup, but using screw-like implants. Threads and machined longitudinal cavities were used as VOIs. Bone formation in these VOIs was compared with intact cancellous bone inside a tube-like VOI around the implants. Stadlinger and colleagues [80] used the cylindrical implant design [78] described earlier to study different biofunctionalized, collagen-based surfaces. Implants were placed in the mandible of a mini-pig. Results of SR micro-CT analysis were in disagreement with histology.

Fiber-reinforced composites (FRCs) are potentially useful alternatives for metallic implants as the mechanical properties of these materials can be tailored to closely match those of bone. As another potential benefit, non-metallic implants may produce fewer artifacts with CT and MRI diagnostic imaging. This high-strength and durable FRC [81] was originally developed for oral use in dental applications. [82, 83] These materials were also tested as load-bearing oral [84-86] and orthopaedic implants. [87] A micro-CT imaging of screw-shaped oral FRC implants with bone was attempted. [88] FRC implants were prepared with and without a bioactive glass coating. Screw-shaped titanium implants served as controls. The implants were placed in the femoral metaphyseal area in the pig. After the retrieval, the bone blocks containing implants were imaged by micro-CT. Bone formation (BV/TV) was studied inside a VOI created inside the implant threads (Figure 13–5). Histological analysis was also performed. As expected, the FRC implants produced less artifacts in micro-CT imaging than did the control titanium implants. Both micro-CT and histology demonstrated the benefit of bioactive glass coatings.

Conclusion

Micro-CT imaging allows for the three-dimensional visualization and quantification of peri-implant bone and is, therefore, a powerful tool for understanding biological reactions around implants. The image data can also be used to perform biomechanical simulation studies of implants in bone, using the FE approach. Recent advances in the development of *in vivo* micro-CT scanners give the possibility to follow-up the same experimental animal at different time points. Therefore, the animal experiments can be refined and the number of animals reduced. Still, due to the imaging artifacts and the insufficient resolution of present micro-CT scanners, an accurate assessment of the direct interfacial contact area between the implant and peri-implant bone is challenging. Histological sectioning still provides superior resolution, and the micro-CT studies should be supplemented by the use of microscopy techniques.

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Chapter XIV

Applications of Electron and Ion Beam Microscopy in Dental Implant Research

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Abstract

Electron and ion beam microscopy techniques are important for the microstructural and analytical characterization of dental implant material systems. Focused ion beam specimen (FIB) preparation techniques offer an increased understanding and ability for 2D and 3D scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of these multi-phase systems. This paper describes usage of and applications for FIB/SEM/TEM in dental implant research.

14.1. Introduction

Relating dental implant properties to the overall characterization of dental implants, including microstructure, ultrastructure, composition, and biocompatible of coatings is essential for the understanding of osseointegration in dental implant research. Electron and ion microscopy are indispensable dental implant research characterization techniques. A scanning electron microscope (SEM) typically operates up to 30 kV and is most useful for imaging surface topography and differences in materials (e.g., phase contrast) or grain sizes. [1] Low SEM accelerating voltages (< 5 kV) are typically used to limit the interaction volume depth and maximize surface information. Higher SEM accelerating voltages (10-20 kV) are used when analytical x-ray results are desired (see below). A scanning/transmission electron microscope (S/TEM) usually operates up to 300 kV and mass/thickness contrast or diffraction contrast effects are used to decipher microstructural and morphological information such as phase boundaries, grain size, interface or interphase structure, and crystalline defects. [2] The

added complexity of S/TEM analysis is the inherent requirement that the specimen be electron transparent in the region of interest. The specimen thickness required is atomic number dependent (i.e., higher Z materials must be thinner for electron transparency) and S/TEM technique dependent, but generally, the specimen must be less than ~ 100 nm.

Over the years, S/TEM specimen preparation has become faster, easier, and more reliable with the use of focused ion beam (FIB) techniques. [3] FIB microscopes typically raster nanometer-sized energetic Ga^+ ion beams (e.g., < 50 keV) for site specific material sputtering (e.g., ion milling). In addition, ion induced secondary electron images may also be collected revealing unique and complementary contrast mechanisms when compared with SEM images. Ion induced secondary ion images are also useful for the collection of complementary analysis. Secondary ions are not susceptible to charging artifacts. In addition, secondary ions can be collected and mass separated using a secondary ion mass spectrometer (SIMS). [4] While FIB/SIMS has seen limited uses, its effectiveness is undeniable and its spatial resolution for minor elements can be on the order of the ion beam size (~ 5 nm).

The modern FIB column is often paired with an SEM column onto a single vacuum chamber producing a dual platform instrument that enhances the capabilities of either column working alone. The synergistic FIB/SEM instrument allows for specimen preparation end-pointing of the FIB milling process via SEM imaging. Combining the sequential and alternating methods of FIB “slicing” with SEM imaging allows the acquisition of stacks of 2D images. This tomographic image stack can be used to create 3D models of dental implant structures. 3D electron tomograms can also be collected using an S/TEM by acquiring multiple 2D images from a tilt series and then performing the back projection analysis. In addition to images that may be collected with the FIB/SEM or S/TEM, electron induced x-rays can be detected in a FIB/SEM or an S/TEM using an x-ray energy dispersive spectrometer (XEDS). XEDS analysis can be used either to identify elemental composition or spectral information at specific points or 2D maps of spectral information can be collected. Below are examples of 2D and 3D characterization of dental implants using FIB/SEM and S/TEM.

Two Nobel Biocare TiUnite (Nobel Biocare, Yorba Linda, CA) dental implants were surgically removed from a human patient because of implant mobility observed upon stage 2 uncovering of the implants. All images were obtained from these failed dental implants unless otherwise mentioned. Instruments used for this work include an FEI Quanta 3D DualBeam equipped with an Oxford XEDS, FEI Strata 400S DualBeam, FEI Helios 400S DualBeam, FEI FIB200 workstation equipped with a SIMSMAP III detector, FEI Spirit BioTwin, and an FEI Tecnai F20 S/TEM equipped with an EDAX XEDS. Details of much of this work may be found elsewhere. [4-6]

14.2. Surface Characterization of Dental Implants with SEM/XEDS

The SEM can be used to image the surface topography of the dental implant. Electron induced secondary electrons (SEs) are low energy electrons. These low energy electrons can only escape very close to the imaged surface. [1] Since the emission of secondary electrons is enhanced at angled and free surfaces, the collection of this signal provides excellent

topographic imaging information. Figure 14–1 shows several SE SEM images of the surface of a dental implant. A low magnification SE SEM image of a dental implant is shown in figure 14–1a. Figure 14–1b shows a region of the implant with no evidence of osseointegration. The coating porosity is evident with pore diameter sizes in the range of 250 nm to 2 μ m. XEDS results presented in figure 14–1c obtained from the coating shows evidence of Ti, O, and P, which is indicative of the coating devoid of any Ca-rich bone formation.

The collection of higher energy backscattered electrons (BSEs) in the SEM will yield less topographic information with more sensitivity to atomic number or Z contrast effects. The BSE signal is also less susceptible to sample charging artifacts. The BSE SEM image in figure 14–2a shows a region of the dental implant thread with evidence of bone growth and apparent osseointegration.

The higher Z Ti-rich coating yields bright contrast while the lower Z Ca-rich bone yields darker contrast in the image. Partial osseointegration is observed in regions where the bright coating pore edges surround the infiltration of bone within the pore structure.

XEDS from a region where bone has partially grown into the implant coating pore structure is shown in figure 14–2b. The presence of all four elements Ca, Ti, O, and P is indicative of x-rays originating from both the bone and the coating.

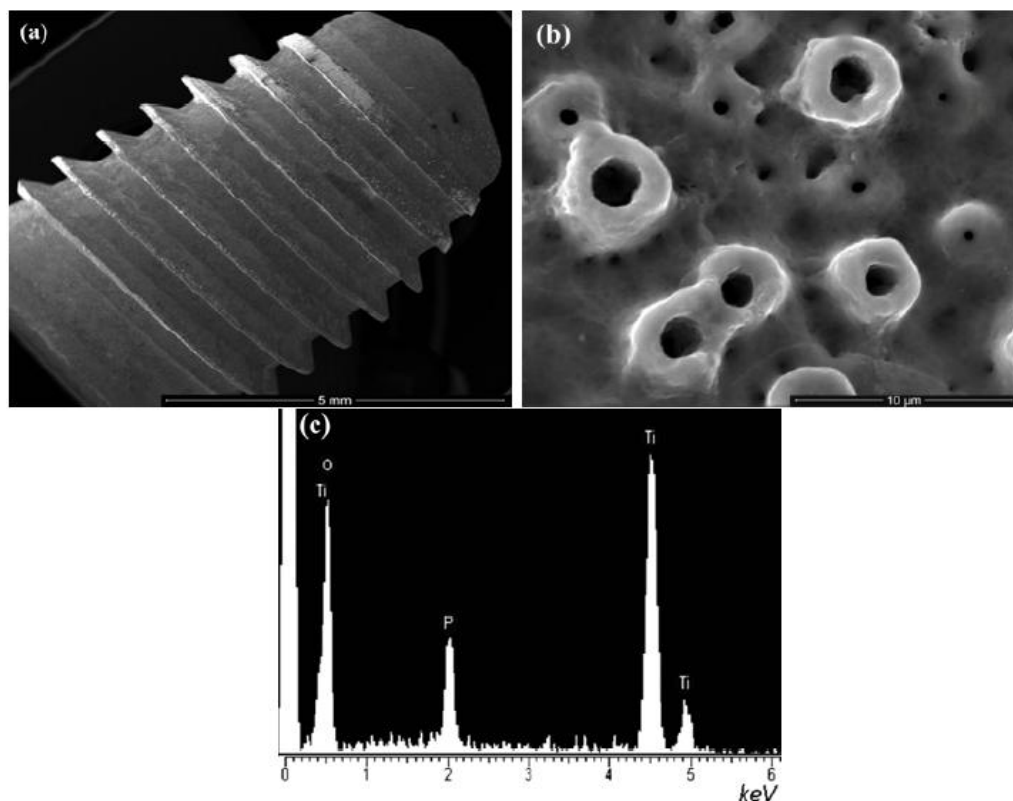


Figure 14.1. (a) Low magnification SEM image of the failed dental implant. (b) SEM image of the porous dental implant coating surface with no apparent osseointegration evidenced by the presence of only the coating constituents Ti, O, and P, as shown by the XEDS results presented in (c).

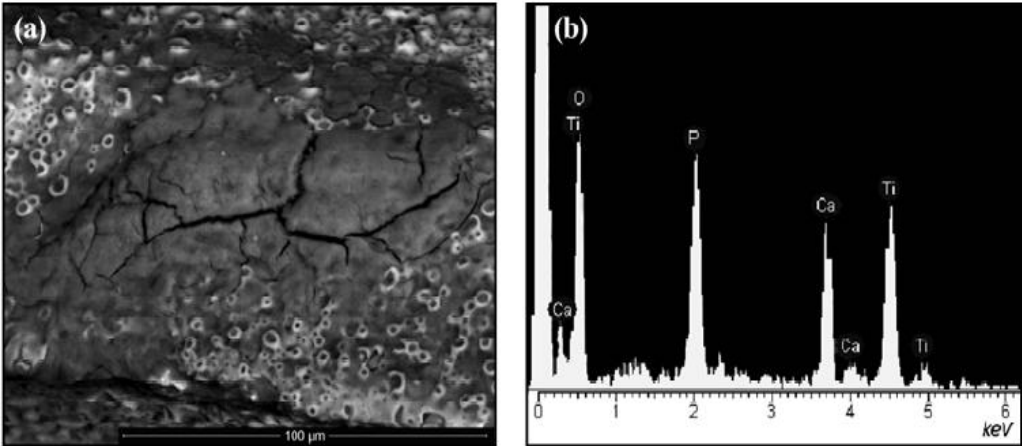
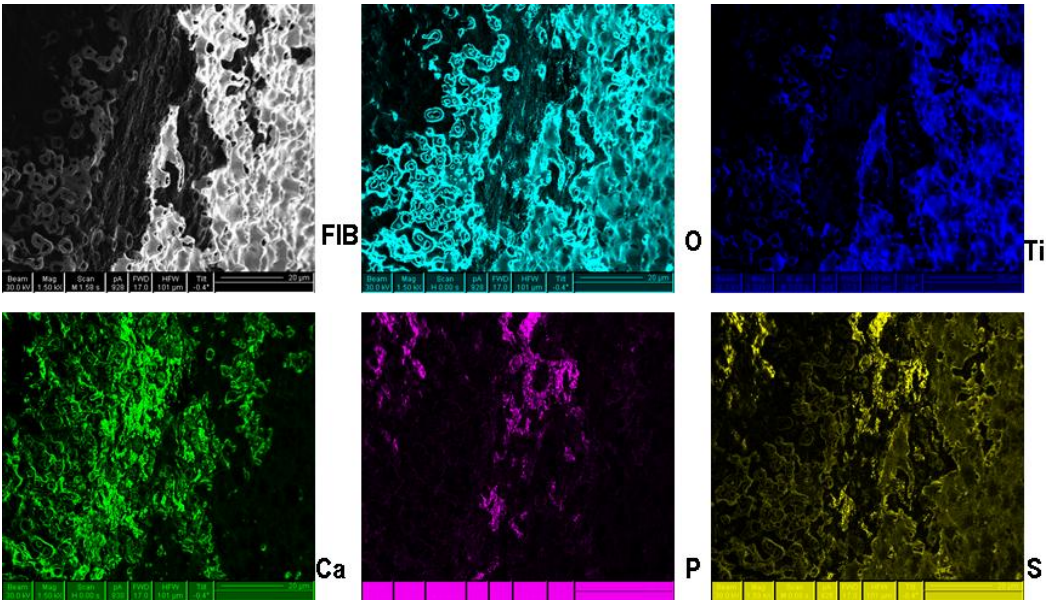


Figure 14–2. (a) BSE SEM image shows bone growth and osseointegration into the pores of the implant thread structure. (b) XEDS results of a bone/coating region showing evidence of Ca, Ti, O, and P.

14.3. Surface Characterization of Dental Implants Using FIB/SIMS

As mentioned earlier, the FIB itself is capable of providing unique contrast through the collection of ion induced secondary electron images with resolution ~ 5 nm. In addition, the secondary ions which are emitted from the ion bombarded target may be collected and mass separated for isotope specificity.



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Figure 14.3. SE FIB image and FIB/SIMS map of O, Ti, Ca, P, and S. The horizontal field width of each image is 100 μm.

The use of Ga^+ ions as a primary ion source yields low secondary ion yields compared to traditional SIMS ion sources. [4] However, the FIB/SIMS spatial resolution rivals XEDS analysis with the added benefit of isotope specific surface trace analysis. Figure 14–3 shows a FIB induced SE image of the surface of a dental implant partially covered with bone. In addition, FIB/SIMS maps of O, Ti, Ca, P, and S are also presented. The FIB/SIMS results yield immediate insight into the spatial distribution of the bone growth on the implant surface at the sub micrometer scale.

14.4. 2D and 3D Characterization of Dental Implants with FIB/SEM

While SEM imaging provides excellent analysis of the surface of the implant and surface area bone osseointegration coverage, it is desirable to expose a cross-section of the layer interfaces (i.e., bone/coating/substrate) to assess the interfacial properties, microstructure, and morphology in the 3rd dimension. Site-specific cross-sectioning may be performed with FIB milling. Details on the basics of FIB instrumentation, physics of ion bombardment, and sample preparation techniques are detailed elsewhere. [3,6] In essence, FIB milling relies on physical sputtering of the target with nanometer sized focused ion beams that can be positioned spatially to within $\sim 10\text{nm}$. This allows for material removal for site specific cross-section FIB and SEM examination of dental implants, and as shown below, for TEM specimen preparation and analysis. Regions of the implant surface with obvious bone growth may be cross-sectioned using the FIB milling techniques.

Figure 14–4 shows a FIB cross-sectioned bone on dental implant imaged using three different signals with similar fields of view. The arrow in each image points to the feature common to all images. Figure 14–4a shows an SE FIB image of the FIB prepared cross-section. Figures 14–4b and 4c are SE and BSE SEM images, respectively. The three images show different contrast mechanisms yielding complementary information. Note the polycrystalline micrometer-sized grain structure in the Ti substrate. The ceramic coating contains nanometer-sized porosity at the Ti substrate/coating interface. The rough and porous coating surface structure is obvious in the cross-sectioned images. Bone growth is observed to partially fill some of the larger and deeper “volcano-shaped” coating pores. These volcano-shaped pores indicated by the arrow in the images are $1\text{--}2\mu\text{m}$ at the surface and open up to $4\text{--}5\mu\text{m}$ extending nearly through the coating thickness of $\sim 3\text{--}5\mu\text{m}$. The shape of these pores facilitates the mechanical locking shear strength of the bone/coating interface which will become evident below when analyzed in 3D. “Open space” is observed between the bone and several regions of the coating surface, indicating that incomplete osseointegration is observed and bone growth is not always in intimate contact with coating. Figure 14–4d shows XEDS results from the specific layers, corroborating the observation and location of the Ti substrate, the Ti, O, and P rich coating, and the Ca-rich bone growth. The small Ga peak is due to the Ga^+ FIB milling procedure.

A dual platform FIB/SEM allows for 3D microstructural analysis of samples by automatically alternating between FIB milling and SEM imaging. This allows for unattended acquisition of a stack of 2D images with a FIB milling slice between images (e.g., the z direction in the tomogram) that can be varied from $\sim 10\text{ nm}$ to several micrometers.

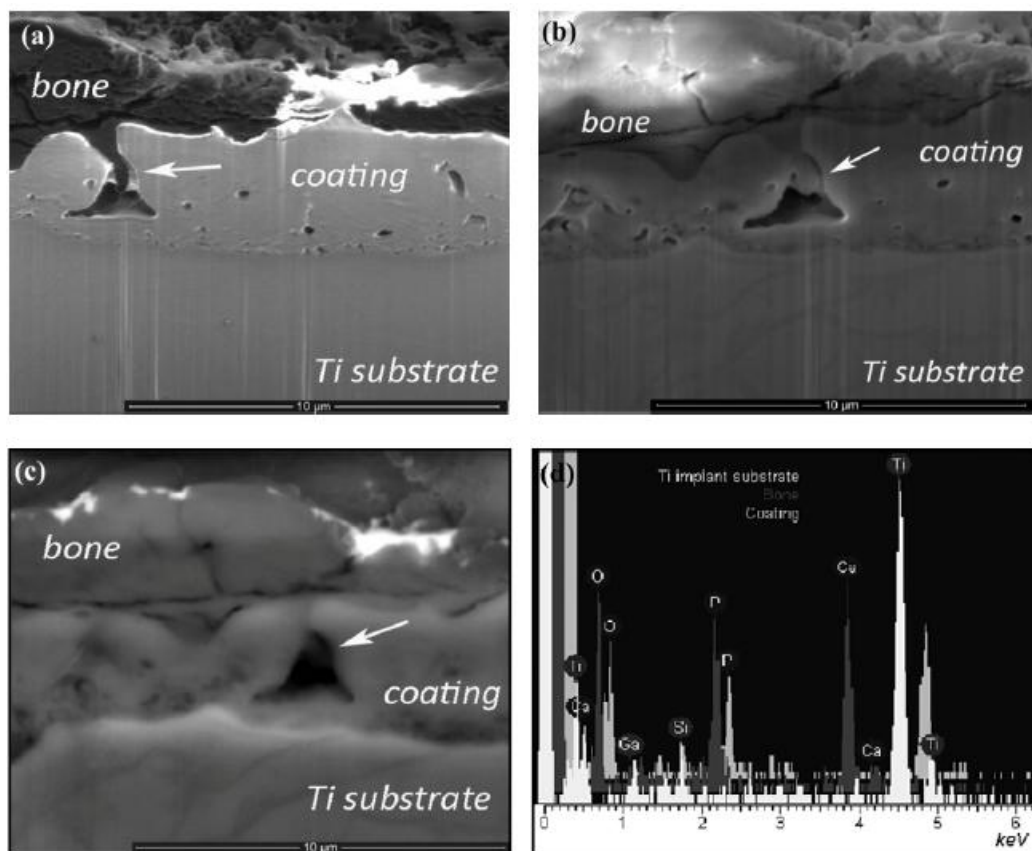


Figure 14.4. Images of a FIB cross-sectioned bone/dental implant revealing interfacial structure and morphology. The bright contrast above the bone in the FIB and BSE SEM images is FIB deposited Pt that is often used in sample preparation. (a) SE FIB image. (b) SE SEM image. (c) BSE SEM image. (d) The XEDS results from the individual layers confirm the interfacial identification.

Thus, the complete voxel dimensions are defined by the image pixel resolution (x, y) and the FIB milling slice thickness (z). In general, for isotropic voxels, it is best to choose the FIB milling slice thickness (z) such that it is on the same order as the image pixel size. In addition, for accurate 3D model reconstruction, a good rule of thumb is to set up the FIB milling slice size such that at least 10 images per feature can be acquired. As an example, if a feature has dimensions of $1\mu\text{m}$, the FIB milling slice should be $< 100\text{ nm}$. Geometric corrections of the y pixel dimension must be accounted for if the SEM images are not acquired at 0° incidence (i.e., normal to the FIB section face).

A subset of 14 BSE SEM images from the entire set of 68 images acquired is shown in figure 14–5a. Each image was obtained after FIB milling 100nm of material away, thus a total segment of $6.8\mu\text{m}$ was sectioned. The images were corrected for sample tilt (in y) and aligned. The horizontal field width of each image is $\sim 25\mu\text{m}$. In this region, the bone is observed to conform to the coating surface and grow in all directions (x, y, z) as shown by the open pore structure of the coating. Because the pores have a volcano-shaped structure as discussed above, any bone that grows into this open pore region may yield a 2D section that shows the presence of bone that appears to be under the coating, as can be seen in multiple slices from the montage in figure 14–5a.

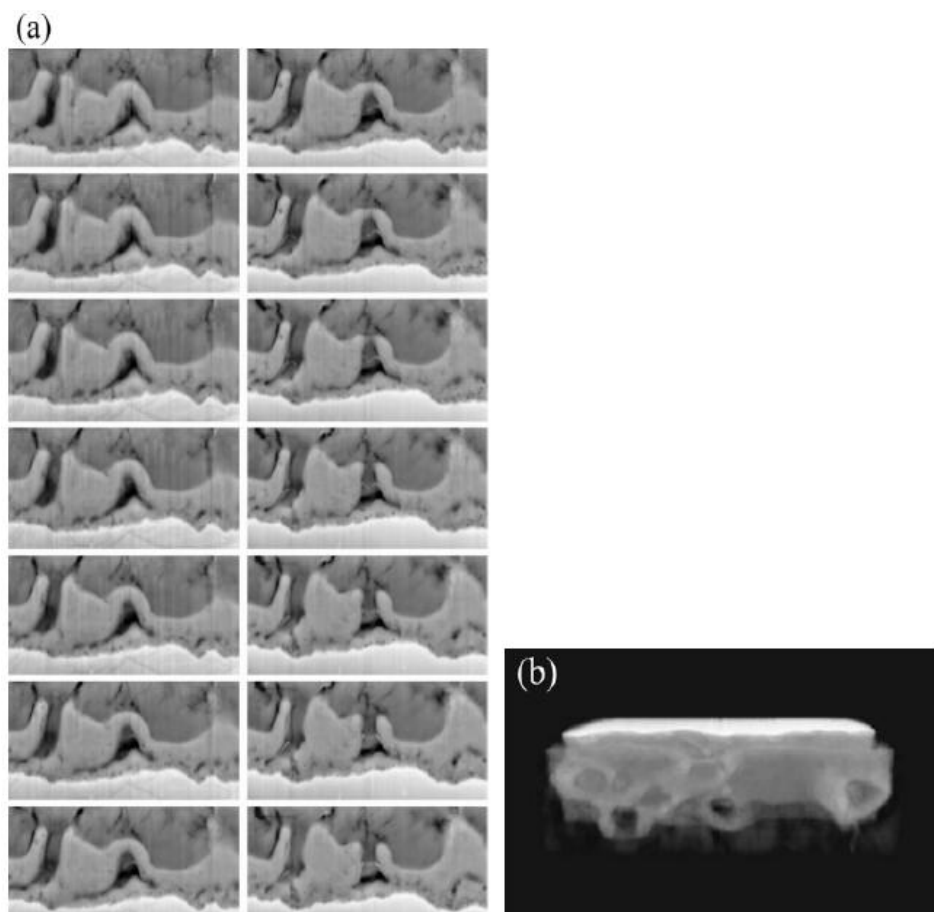


Figure 14.5. (a) Montage of a subset of 14 BSE SEM images obtained using a FIB slice of 100nm between images. (b) 3D model of reconstructed stack of 68 images. The horizontal field width of each image is $\sim 25 \mu\text{m}$.

This illustrates the usefulness of 3D characterization of dental implant systems and is additional evidence for the mechanical locking mechanism of the bone/implant structure. The stack of images can then be rendered for 3D modeling in a number of ways. Orthogonal sections, volume rendering, or surface rendering can be visualized. Figure 14–5b shows a volume reconstruction viewed at a slight angle with respect to the xz acquisition plane. Note that the bone located at the bottom of the image is observed at partial transparency. The bright region in the top of the image is the Ti substrate. The volcano-shape of the coating is obvious in this 3D model where the coating and bone growth into the coating is evident.

14.5. TEM of Dental Implants

FIB and FIB/SEM methods for TEM specimen preparation have been well documented over the years. [3] These TEM specimen preparation methods were first applied to dental implants by Giannuzzi et al., [5,6] and since then used in other publications for TEM studies on dental implants. [7-9] FIB techniques allow for site-specific sectioning of hard and soft

materials within the same section devoid of mechanical damage often incurred in ultramicrotomed prepared specimens. [3].

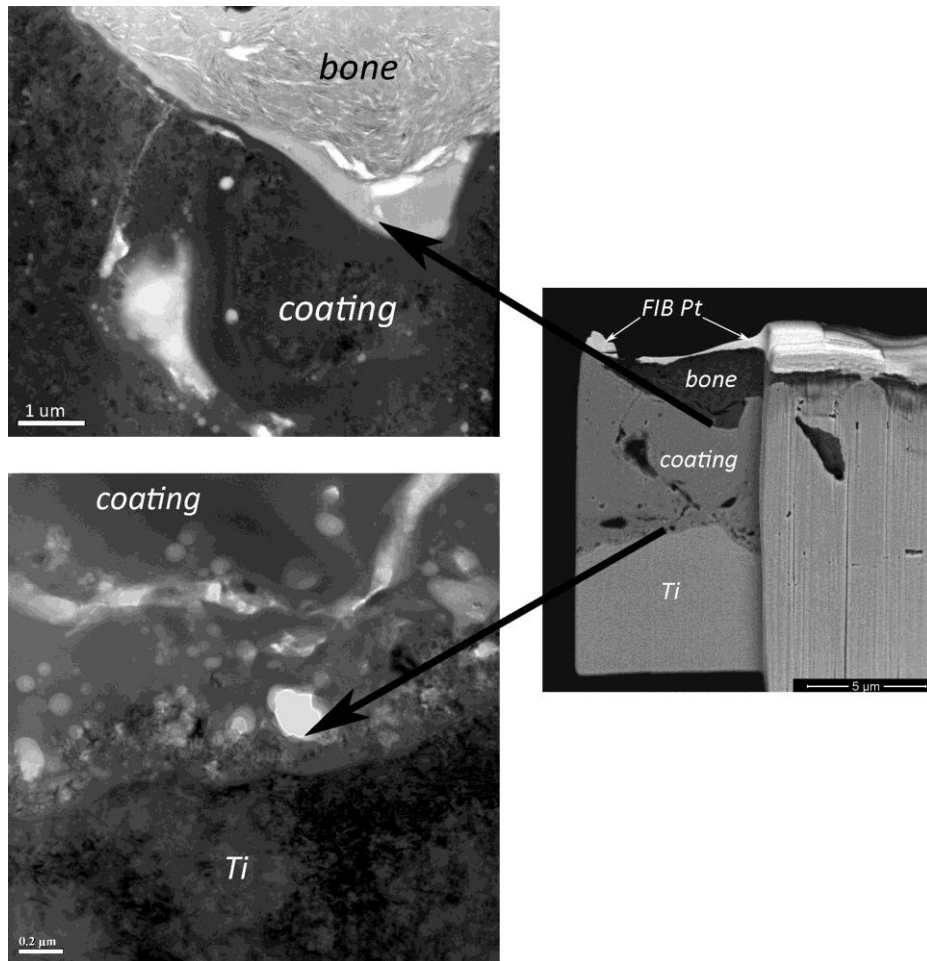


Figure 14.6. (Right) A BSE SEM image of a FIB prepared specimen. (Left) TEM images from FIB prepared specimen.

Figure 14–6 shows a BSE SEM image (right) of a FIB thinned specimen showing the bone/coating/Ti substrate interfaces. Also in figure 14–6 are TEM images (left) obtained from this FIB prepared specimen. There are numerous microstructural features observed from both TEM images that are not discernable in the SEM image. Note the large dislocation density observed via the TEM image within the Ti substrate. Nanoporosity and nanocrystallinity is observed within the coating at the coating/Ti interface. An amorphous phase is observed between the crystalline bone structure and the coating. The relative size of this amorphous phase varies along the bone/coating interface. Note that the bone is observed in its “natural” state, i.e., no staining is required to observe the crystalline diffraction contrast. It was previously shown that interdiffusion of Ca, P, and O occurs at the bone/coating interface, indicating that chemical bonding as well as mechanical bonding occurs at this interface. [6].

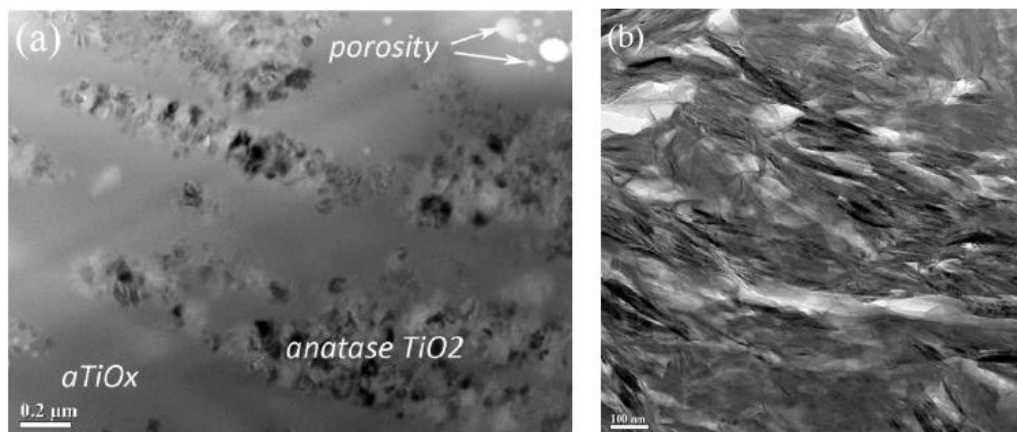


Figure 14.7. (a) TEM image of the coating. (b) TEM image of collagen within the bone.

Figure 14–7 shows details of the (a) coating and (b) the bone structure. The dual phase composition of the coating is evident in figure 14–7a showing both an amorphous TiO_x (aTiO_x) phase as well as nanocrystalline anatase TiO_2 . Figure 14–7b shows the bone structure in detail with evidence of nanocrystalline collagen structure.

14.6. 3D S/TEM Tomography

A recent report by Grandfield et al. [9] used FIB specimen preparation techniques to analyze a bone/hydroxyapatite interface using STEM tomography. The single axis tilt series technique was used to visualize the 3D collagen banding arrangement at a hydroxyapatite (HA) scaffold and human bone interface. They found that the HA crystals in the bone far from the bone/scaffold interface (i.e., greater than ~ 100 nm) are aligned parallel to the scaffold surface. However, the HA crystals of the precipitated apatite at the bone/scaffold interface appeared to be strongly oriented perpendicular to the scaffold surface. The direction of the collagen banding was consistent with the growth direction of the HA crystals.

Conclusion

Electron and ion beam microscopy techniques yield crucial microstructural and compositional information from dental implant material systems. FIB specimen preparation techniques offer an increased understanding and ability for 2D and 3D SEM and TEM on these multi-phase systems.

Acknowledgments

I wish to thank my brother Nicholas J. Giannuzzi, DDS, for helpful comments and discussion. Mario Capuano, DDS, is gratefully acknowledged for supplying the dental

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Chapter XV

Molecular Biology Techniques

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Abstract

In order to better facilitate wound healing and osseointegration following implant placement, an understanding of the inter-relationship between the molecular events involved in these processes is critical. A wide range of techniques commonly grouped under the banner of ‘molecular biology’ are now available to the researcher, allowing interrogation of the three key molecular processes that ultimately lead to clinically observed osseointegration i.e. DNA, RNA and protein expression. This understanding may then be translated into clinical practice by providing leads for further implant development based on a sound understanding of the osseointegration process at the molecular level. This chapter therefore aims to give the reader a brief overview of the various molecular biology techniques that are used extensively in the research laboratory today with specific examples of how they are being applied to the examination of the biological processes regulating osseointegration.

15.1. Introduction

The observable characteristics or traits of a cell or organism, i.e. it’s morphology, development, biochemical and physiological properties, behavior etc, is known as the phenotype, which is the result of the expression of an organism’s genes (as well as environmental factors). This is different from an organism’s genotype, which is the inherited set of instructions that it carries within its genetic code. It is this genetic code, made up of DNA, which in response to stimuli or instruction is transcribed to messenger RNA (mRNA), which is subsequently translated to protein that ultimately determines the phenotype of an organism.

Successful osseointegration of dental implants therefore is determined by the complex interaction of multiple biological systems described by a subject's phenotype i.e. biochemical, hormonal, cellular, immunological, etc. Fortunately, the (continuing) technical developments in molecular biology witnessed over the previous two decades, has provided researchers with the tools to not only identify, but to also manipulate at the level of the genome, some of the critical aspects in the osteogenic process essential for healing clinical osseous defects.

Molecular biology techniques can be broadly described as those methods used to examine the interrelationship and regulation of DNA, RNA and proteins. Most of the techniques in common use today can trace their origins back to the seminal research by Kary Mullis in the mid 1980's on the properties of the DNA polymerase from *Thermus aquaticus* (Taq) that allowed the amplification of small quantities of DNA into useful amounts via the polymerase chain reaction (PCR). Subsequently, this has now made the investigation of the detailed dynamics of gene expression during bone healing and osseointegration an achievable goal.

15.2. DNA Expression

Genetic differences (e.g. single nucleotide polymorphisms, large and small scale mutations etc.) inherent between individuals are often reflected in their susceptibility to disease. As the fundamental building block of the genome, analysis of DNA via techniques such as PCR allows the determination of genetic differences between individuals. Subsequently, the association of such genetic differences with disease expression give insights into the role of genetic diversity in a given disease state. For example, Kornman et al (1997) demonstrated using real-time PCR, [1] that subjects with a specific IL-1 genotype had higher levels of the pro-inflammatory cytokine IL-1 β and more severe periodontitis (odds ratio 18.9) as measured by pocket depth, attachment and bone loss. More recently, polymorphisms of the IL-1 gene cluster have also been associated with peri-implantitis, [2] an inflammatory reaction affecting the tissues surrounding osseointegrated dental implants resulting in loss of supporting bone. [3,4] In their study, an association was found between the IL-1RN gene polymorphism and peri-implantitis (odds ratio 3.0). Similarly, IL-1 gene polymorphism at IL-1A-889 and IL-1B+3954 have been demonstrated to adversely affect treatment outcomes of peri-implantitis in genotype positive individuals. [5] Although a recent review of the literature [6] concluded that the association between the IL-1 genotype status and peri-implantitis is still unresolved, these studies serve to demonstrate the ability of molecular biology techniques such as real-time PCR to help elucidate the biological mechanisms underlying disease processes likely to affect the long term success of osseointegration.

Molecular biology methods based on the DNA sequence evolved from the original work of Southern (1975) and typically include hybridisation techniques using DNA probes (checkerboard platform) and *in situ* hybridisation with labelled oligonucleotide probes to identify and quantitate complementary DNA sequences. [7] DNA amplification techniques such as the polymerase chain reaction (PCR) and in particular real-time PCR using specific primers however has revolutionised these analyses, freeing the investigator from the limitations of sample material, allowing high throughput at low cost and most importantly, markedly increasing assay sensitivity and specificity.

15.2.1. Checkerboard Analysis

The DNA Checkerboard analysis was initially developed to study the subgingival microorganisms involved in periodontitis. [8] This technique is based on the simultaneous hybridisation of multiple DNA probes to DNA sequences from multiple test samples fixed to a membrane. Currently, the simultaneous and quantitative analysis of up to 28 plaque samples against 40 microbial species can be achieved in a single assay making it ideal for epidemiological studies.

The DNA probes are constructed using the entire genome of a bacterial species as the target and hence this may increase the probability of cross-reactions between species because of common regions of DNA among closely related species. Similarly, whole genomic DNA probes might not detect all strains of a given species. The technique however is rapid, sensitive, and relatively inexpensive. It also overcomes many of the limitations of cultural microbiology including loss of viability of organisms during transport and the problem of enumerating / speciating difficult to cultivate species. The DNA checkerboard technique does have limitations. The technique can detect only species for which DNA probes have been prepared. Thus, novel pathogens that might be detected in culture or by other molecular techniques would not be detected by this method.

Checkerboard analysis has been utilised in a number of studies to determine the relationship(s) among bacterial species comprising the biofilm found in peri-implantitis. During peri-implant breakdown a complex microbiota is established, closely resembling that found in adult periodontitis. [9-11] Checkerboard analysis of the peri-implant microbiota, including the known periodontal pathogens *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythus* and *Treponema denticola*, in peri-implantitis and implant mucositis sites has been used to not only evaluate the species present, [12] but to also monitor the effect of mechanical and antibiotic therapies. [13,14] This technique has also proved to be useful in following the temporal microbial dynamics from initial colonisation of 'pristine' peri-implant pockets [15] through to long term (10years) clinical and microbiological outcomes. [16].

15.2.2. Polymerase Chain Reaction

The polymerase chain reaction would arguably be the most utilised technique for DNA analysis and hence the interrogation of the genome today. PCR allows the detection of relatively minor amounts of DNA by amplifying a given DNA sequence using specific primers. A diagrammatic representation of the denaturing, annealing and elongation steps that constitute the classical standard PCR procedure is shown in Figure 15–1.

Although conventional PCR does not allow precise quantitation of the amount of starting DNA, this is readily achievable using real-time PCR (rtPCR). Real-time PCR has the ability to detect PCR products as they accumulate in "real-time" rather than estimating the amount of target accumulated after a fixed number of cycles (as in conventional PCR). For example the TaqMan® process achieves this by utilising a fluorogenic probe designed to anneal to a specific sequence of template between the sense and antisense primers (Figure 15–2).

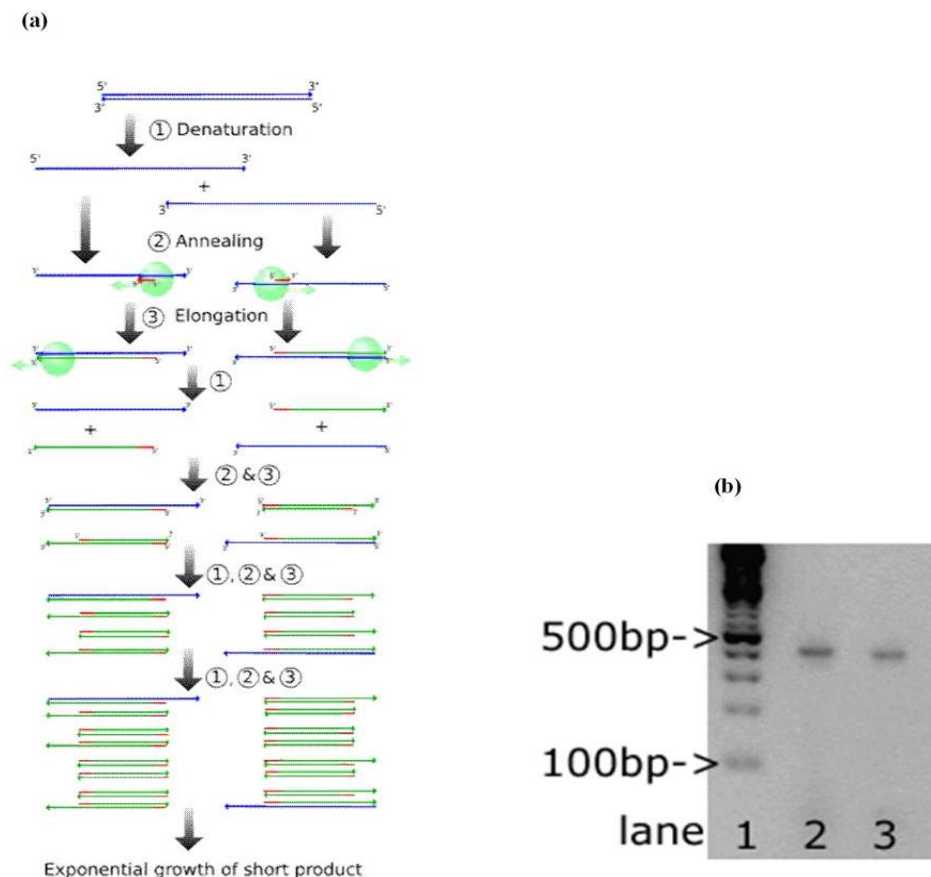


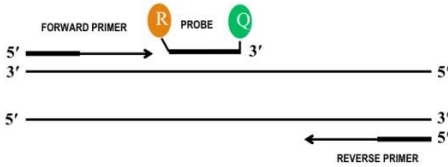
Figure 15.1. (a) Classical steps involved in the polymerase chain reaction and (b) visualisation of the amplified product using agarose gel electrophoresis (Lane 1 100 base pair DNA marker, Lanes 2 and 3 amplified product).

This oligonucleotide probe is constructed to contain a reporter fluorescent dye on its 5' end and a quencher dye on the 3' end that disrupts any observable signal from the reporter dye when it is within a short distance of the reporter dye. Subsequent cleavage of the probe by the 5'-exonuclease activity of the DNA polymerase during the extension phase of the PCR process separates the reporter dye from the quencher dye, increasing the reporter dye signal. Alternatively, the SYBR® Green chemistry uses SYBR® Green I dye, a highly specific double-stranded DNA binding dye which detects amplicon as it accumulates during the PCR cycles (Figure 15–2).

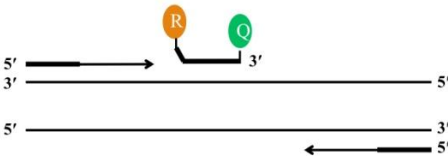
In both cases, the rtPCR reaction is characterized by the point in time during cycling when amplification of the target is first detected (c.f. end-point in conventional PCR). Hence, the higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. Therefore compared to conventional PCR, rtPCR has the following advantages: (a) data is collected in the exponential growth phase, (b) the increase in reporter fluorescent signal is directly proportional to the number of amplicons generated allowing quantitation, (c) increased dynamic range of detection and (d) no-post PCR processing.

Taqman PROBE-BASED ASSAY CHEMISTRY

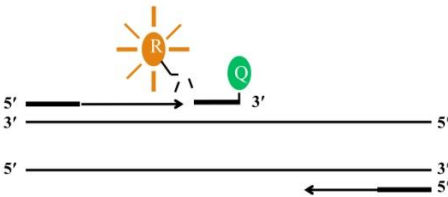
1. Polymerisation: A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a Taqman probe respectively.



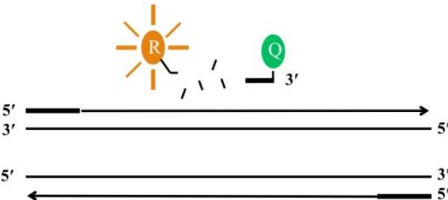
2. Strand displacement: When the probe is intact, the reporter dye emission is quenched.



3. Cleavage: During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



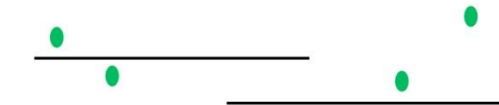
4. Polymerisation completed: Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

**SYBR GREEN 1 DYE ASSAY**

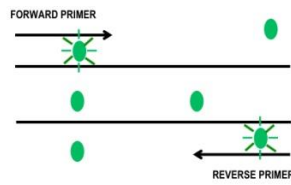
1. Reaction setup: The SYBR Green 1 Dye fluoresces when bound to double-stranded DNA.



2. Denaturation: When DNA is denatured, the SYBR Green 1 Dye is released and the fluorescence is drastically reduced.



3. Polymerisation: During extension, primers anneal and PCR product is generated.



4. Polymerisation completed: When polymerisation is complete, SYBR Green 1 Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected.

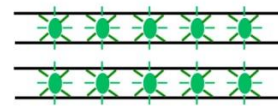


Figure 15.2. Taqman® and Sybr Green® PCR workflows.

An example of the wide scope afforded by real time PCR techniques in the analysis of DNA in implant related research is demonstrated by the detection of bacteria, [15,17] identification of specific gene polymorphisms [2] and the analysis of specific gene expression [18] associated with peri-implant diseases such as peri-implantitis.

15.3. RNA Expression

The analysis of RNA expression, and in particular the expression of mRNA, is important as this is the first step involved in the response of a cell or organism that ultimately results in the production of a protein which will determine the phenotypic response. For example, the placement of an implant will elicit a large biological cascade resulting in the transcription of

thousands of mRNA molecules from genomic DNA, ultimately resulting in the production of thousands of proteins which will determine the nature of the response to implant placement. As the intermediary ‘messenger’ which results in the expression of a ‘phenotype’ from a set ‘genotype’, the detection and quantification of mRNA is a fundamental tool in molecular biology.

Obtaining high quality intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments including rtPCR and microarray analysis. As immediate RNA isolation is not always possible, we recommend the use of RNA stabilization solutions added to the experimental sample to allow the researcher to postpone RNA isolation without sacrificing the integrity of the RNA (e.g. RNeasy®). When purifying RNA, the source of the sample as well as the purification method also needs to be considered. Hard tissue like bone and teeth for example can be problematic, as it is sometimes difficult to ensure the denaturant is in contact with the cellular contents when the cells are disrupted. The most commonly used RNA purification methods use guanidine isothiocyanate / phenol / chloroform extraction alone or in combination with silica gel-based membranes in spin-columns which selectively bind RNA. Affinity chromatography can also be used to increase the relative amount of mRNA able to be isolated from samples.

Following purification, assessment of the integrity and quality of the RNA is essential prior to any further down-stream processing. As mRNA only comprises 1-3% of the total RNA routinely collected, its integrity is usually assessed indirectly by examining the integrity of the major ribosomal RNA (rRNA) species (18S and 28S for mammalian rRNA) using gel electrophoresis. Contaminants co-purified in RNA samples may also interfere with down-stream enzymatic applications such as reverse transcription. Similarly genomic DNA contamination may also introduce a background signal in an rtPCR assay.

15.3.1. Real Time PCR

Reverse Transcription, coupled with the polymerase chain reaction, has literally revolutionized the study of gene expression. It is now possible to detect the RNA transcript of any gene, regardless of the amount of starting material or the relative abundance of the specific mRNA. Theoretically, a single copy of a message can be detected by this technique although in practice, tens to hundreds of copies are required for reliable quantitation. As the PCR process requires a DNA template, reverse transcription of RNA to complementary DNA (cDNA) using a reverse transcriptase is essential. Good RNA quality is essential for the success of these applications because degraded RNA or RNA that contains impurities will perform poorly in most enzymatic applications. Genomic DNA, a common contaminant in total RNA preparations is relatively easily removed by DNase treatment of RNA samples during or after isolation. Gene expression data must also be corrected for any differences in cellular input, RNA quality or reverse transcriptase efficiency between samples. This is usually achieved by normalizing the data against the expression of known reference genes (e.g. B2MG, HPRT1, GAPDH and ACTB) using the $\Delta\Delta C_t$ method. [19].

Quantitative PCR is currently the method of choice to assess the expression of known markers of osteogenic differentiation such as alkaline phosphatase, bone sialoprotein, osteocalcin and osteopontin in various cell types including osteoblasts, mesenchymal stem cells and periodontal ligament fibroblasts both *in vitro* and *in vivo*. [20-23] Similarly, the

analysis of other associated mechanisms involved in bone homeostasis such as the modulation of osteoclastogenesis and osteoclast activity [24] or activation / regulation of osteogenic signalling pathways [25] is also possible using this technique. To simplify matters further, commercial real-time PCR gene arrays are now readily available. These focused gene panels can be used to analyse the regulation of up to 84 genes associated with any one of over 100 biological pathways or specific disease states simultaneously.

15.3.2. Microarray Analysis

Gene expression profiling using microarrays allows the assessment of the full genetic profile of a given sample. It has been utilized *in vitro* to study the response of cultured cells to different implant surfaces as well as to evaluate models of osteoblast differentiation and response to growth factors. For example previous studies as well as our own [20,26] have shown that titanium surface modification may affect osteoblast cell function via TGF β /BMP as well as Wnt signalling.

Although *in vitro* assessment of cell-implant interaction has provided valuable information about the factors that influence osseointegration, the complex nature of this process makes it difficult for key *in vivo* interactions and mechanisms to be studied using *in vitro* models. In reality, *in vitro* studies are only capable of assessing the interaction of one cell type (e.g. osteoblasts) with the implant surface, whereas the true *in vivo* situation involves a wide variety of cells. Ideally, a controlled *in vivo* model would be the most clinically relevant approach to study the process of osseointegration.

Microarrays have also been utilized in studying the gene pathways involved in bone healing and regeneration *in vivo*. Hadjiargyrou et al (2002) utilized microarrays to assess changes in gene expression over 21 days in a rat femur fracture model. [27] They were able to demonstrate that a large number of genes (588 known genes and 821 expressed sequence tags) were differentially expressed between the healing bone and the control healthy femurs. They concluded that the healing process is exceedingly complex and that groups of genes rather than individual molecules need to be considered if the regeneration of bone is to be accelerated exogenously. Furthermore, Clancy et al (2003) utilized microarrays to study changes in expression over a 14 day period in BMP-2 induced endochondral bone formation. [28] The majority of the gene expression changes occurred in the first 7 days, and the authors concluded that “arrays enabled a broader view of endochondral bone formation that has been reported to date”.

We have also recently examined the temporal gene expression profile associated with the early healing events during osseointegration in a human model using microarrays. Using this whole genome approach, we could demonstrate that temporal transcriptional changes during osseointegration involve the expression of proliferation and immuno-inflammatory response associated genes during the early stages of osseointegration which are ultimately replaced by genes associated with the biological processes of skeletogenesis, angiogenesis and neurogenesis. The early immuno-inflammatory changes appear to be regulated via the I κ B kinase/NF κ B cascade, while the later osteogenesis related mechanisms are regulated by TGF β /BMP, Notch and Wnt signalling. [29] Gene profiling therefore allows an insight into the specific healing-associated genetic pathways which lead to osseointegration.

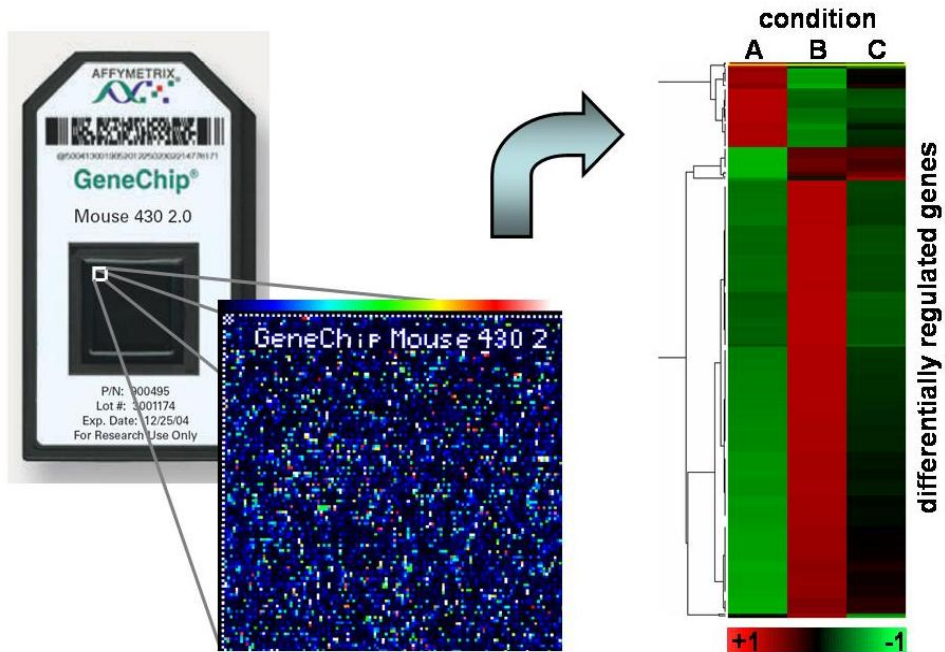


Figure 15.3. Affymetrix GeneChip® microarray system.

Furthermore, the combination of histological analysis following the use of established experimental *in vivo* models with the genetic pathways of early healing events will allow direct correlation between molecular and clinical events during early healing.

Hybridization between sample DNA (in this case cDNA transcribed from RNA) and DNA probes is the same basic principle behind gene expression profiling using microarrays as previously described for checkerboard analysis. In the most commonly used microarrays, the probes are attached to a solid surface such as a silicon chip (Affymetrix) or microscopic bead (Illumina). A key advantage of these high-density arrays is the ability to provide multiple, independent measurements for each transcript. The Affymetrix microarray for example is comprised of more than 54,000 probe sets which include over 38,000 well-characterized human genes (Figure 15–3). The Illumina HumanHT-12 v4 Expression BeadChip also targets more than 47,000 probes. Following the incorporation of biotin-labelled nucleotides by *in vitro* transcription amplification of the sample cDNA, hybridization to the probes is quantitatively assessed by measuring the fluorescence emission of bound streptavidin-Cy3 stain. DNA microarrays can thus be used to measure changes in RNA expression levels, to detect single nucleotide polymorphisms (SNPs), or to genotype or re-sequence mutant genomes.

15.3.3. RNAi

Post-transcriptional gene silencing is a powerful, recently developed tool used in the analysis of gene function. This technique takes advantage of the intrinsic RNA interference (RNAi) system that acts in an epigenetic manner to regulate gene activity. In this system, small RNA molecules i.e. microRNA's (miRNA) which are encoded in the genome or small

interfering RNA's (siRNA) produced by enzymatic cleavage of long double-stranded RNA (dsRNA) or short hairpin RNA (shRNA), can be incorporated into the RNA-induced silencing complex (RISC) where they act as templates for complementary sequences of messenger RNA. Hybridisation subsequently induces RNA cleavage by argonaute (Ago), the catalytic component of the RISC complex and hence inactivation or silencing of the gene's activity. Experimentally therefore, the introduction of specific dsRNA sequences into cells both *in vitro* and *in vivo* may be used to induce suppression of specific genes of interest via RNAi.

Indeed research is now appearing in the literature using siRNA technology to investigate further the mechanism(s) of action of genes known to play a role in osteogenesis. Olivares Navarrete et al (2010) for example have demonstrated silencing of the $\alpha 2\beta 1$ integrin gene in osteoblasts cultured on microstructured titanium inhibits the secretion of Dickkopf-2 (DKK2) that normally acts in a paracrine manner to induce mesenchymal stem cell differentiation towards an osteoblastic lineage. [30] Further work from this group has also shown that silencing the $\alpha 2\beta 1$ integrin subunit mediates secretion of the angiogenic growth factors VEGF-A and FGF-2 by osteoblasts. [31].

15.3.4. *In Situ* Hybridization

In situ hybridization (ISH) is a hybridization technique that uses labelled (e.g. digoxigenin, biotin, tritium etc.) complementary DNA (cDNA) or RNA to localize a specific DNA or RNA sequence in a portion or section of tissue. Finding the optimal balance between good morphological preservation of cells and strong hybridisation signals is crucial. Care also needs to be taken to ensure that the cDNA probes do not match any common housekeeping gene sequences for cellular proteins.

Although transcriptional profiling e.g. using microarrays of tissues associated with clinically relevant osseointegration provides a unique opportunity to study the multiple overlapping events that occur during osseointegration, it also has its limitations. Because the tissue which is obtained contains a mixture of cell types, it is often not possible to attribute specific biological mechanisms to individual cell types. It is also not possible to localize the source of the gene expression within the healing wound, which could only be achieved with *in situ* hybridization. ISH therefore allows the modulation of gene expression in cells interacting with the prosthetic surface to be demonstrated e.g. Goodman et al (1998) for example has shown the importance of T-cell modulation and macrophage function in implant loosening and osteolysis by ISH localisation of the expression of the pro-inflammatory cytokines IL-1, IL-6 and TNF- α . [32].

15.4. Protein Expression

To fully understand biological processes, transcriptional changes in gene expression need to be confirmed as resulting in a corresponding change in protein expression as these molecules are the ultimate effectors determining phenotype. Unfortunately, the expression of mRNA for a given protein is not always directly proportional to the amount of protein which

is subsequently produced. Furthermore, one must take into accounts that whereas DNA and RNA have set specific codes, proteins tend to be subject to extensive post-translational modification which can greatly influence their function. A number of techniques are routinely utilised in determining protein expression.

15.4.1. ELISA

Immunological methods such as the enzyme linked immunosorbent assay (ELISA) are designed to take advantage of the specific nature of antibodies. The normal polyclonal immune response to the presence of antigen, where a number of antibodies are produced which recognise different epitopes present on the antigen, is of limited use in clinical laboratory assays, as the same epitope on different target antigens would result in non-specificity. In the early 1970's the development of techniques for producing monoclonal antibodies (MAb) i.e. one cell line producing the same antibody of known specificity, revolutionised the use of immunological methods in both clinical and laboratory research. Spleen cells from an animal previously vaccinated with the antigen under consideration are fused with myeloma cells to produce a 'hybridoma'. These immortal antibody producing cell lines are then screened for antigen specificity and usually conjugated to a reporter molecule (enzymatic / fluorescent). The resulting assay combines the specificity of antibodies (particularly monoclonal antibody) with the sensitivity of simple enzyme assays. Some limitations of this technique include that the immunoreactivity of the primary antibody may be reduced as a result of labelling and there is little signal amplification limiting assay sensitivity. To overcome some of these limitations, the indirect or 'sandwich ELISA' has been the most widely used variation where two antibodies directed at different epitopes on the same antigen are often utilised.

In a recent study we have demonstrated using ELISA that the secretion of BMP-2, a protein known to affect the proliferation and differentiation of osteoblasts, was significantly up-regulated in osteoblasts cultured on a hydrophilic titanium surface. [20] Dental implants constructed of this material have been demonstrated clinically to show superior osseointegration characteristics. This technique confirmed our microarray analysis that showed BMP-2 had the largest fold change increase in expression with culture on the hydrophilic surface. BMP-6 gene expression however, which was also demonstrated to be significantly up-regulated at the transcript level, could not be detected by ELISA. This may have been because it was not translated to a secreted protein, was below the threshold of detection of our method or was subject to post-translational modification that rendered it undetectable by our specific antibody.

15.4.2. Immunohistochemistry

Immunohistochemistry, much like ELISA, also takes advantage of the specific nature of antibodies to determine the distribution, localization and expression of proteins in tissue sections prepared from biological specimens. Proteins, cytokines etc within the tissue of interest then act as antigens against which specific antibodies have been raised in an experimental animal. As a result these immunohistochemical reactions can be very precise.

The specific antigen-antibody interactions then need to be visualized using a marker such as fluorescent dyes, enzymes or radioactive elements for example that have been bound to the antibody (Figure 15–4). In a commonly used variation of this technique, amplification of the signal (i.e. antigen-antibody interaction) may be achieved by utilising a second labelled antibody to bind to the specific (primary) antibody bound to the target antigen. The first ‘direct’ method whilst specific and reduces the likelihood of background noise, does have the disadvantage of having to label the primary antibody first which may interfere with assay sensitivity. The second ‘indirect’ method is generally more sensitive and versatile as a variety of primary antibodies can be used with the same conjugated second antibody. There are numerous immunohistochemistry methods however that may be used to localize specific antigens and as such the selection of a suitable method should be based on parameters such as the type of specimen under investigation and the degree of sensitivity required. This approach therefore can be used to provide visualisation of the events taking place at the interface between an implant and the surrounding tissue. This is of particular importance given the current intense scientific interest in the physical and/or chemical modification of implant surfaces aimed at inducing specific cell and tissue responses.

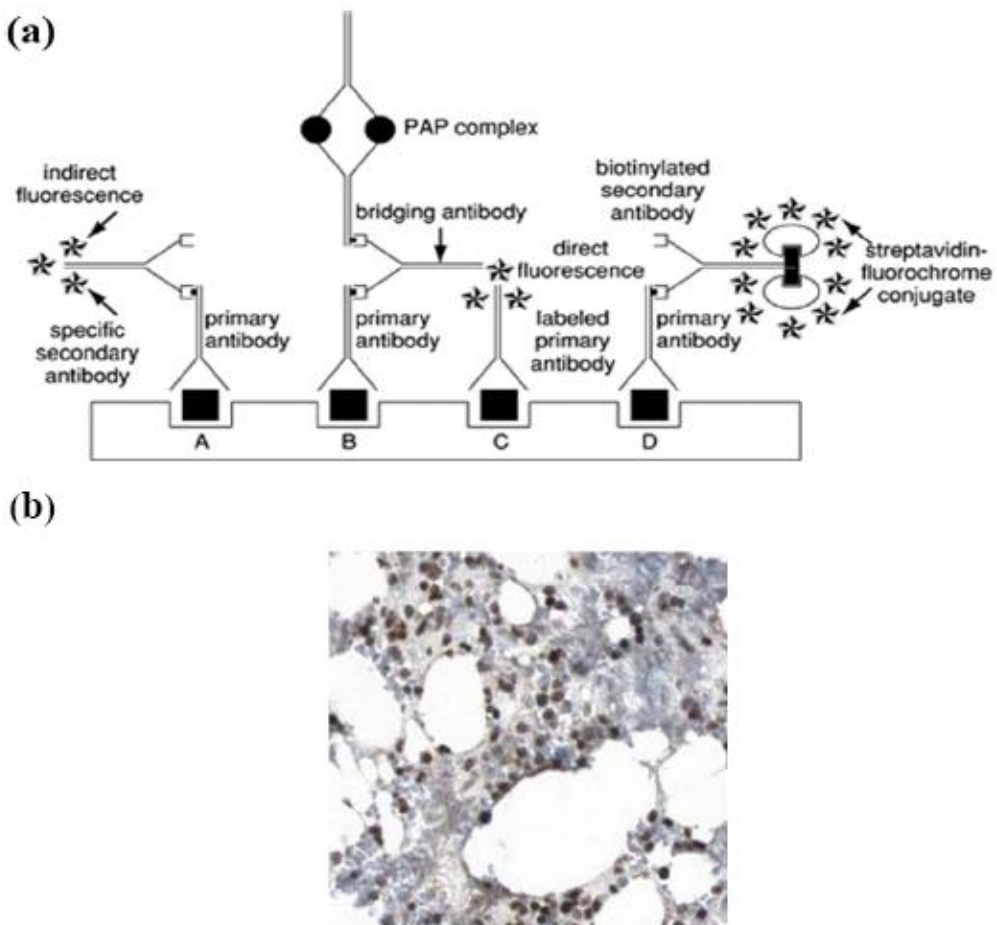


Figure 15.4. (a) Methods of immunohistochemical staining. (b) Paraffin embedded human bone marrow tissue stained for Sp7 / osterix.

Immunohistochemical analysis of osseointegration however has generally proven difficult because specimens are usually not decalcified and embedded in resin, which does not lend itself to routine preparation for immunohistochemical analysis. However, there have been examples where this technique has been used. Immunohistochemical analysis was used as one of the first methods to give insight into the molecular mechanisms occurring at the implant—tissue interface by demonstrating the expression of the bone associated attachment proteins osteopontin and osteocalcin during osseointegration. [33] Recently, Schwartz et al (2008) were able to identify the angiogenesis marker transglutaminase II during osseointegration using immunohistochemistry. [34].

The analysis of soft tissues using immunohistochemistry is far easier than the analysis of mineralized tissues as these can be readily embedded in paraffin, and it is easier to prepare quality sections than is the case with mineralized tissues. The analysis of immunoinflammatory and connective tissue associated proteins in peri-implant tissues has been undertaken using immunohistochemistry. [35].

A closely related technique to immunohistochemistry is immunocytochemistry, whereby the expression of specific proteins is localised in cells grown *in vitro*, rather than histological sections of *in vivo* material. This method has been used to assess the protein expression of adhesion and differentiation proteins in response to different titanium surfaces. [36].

15.4.3. Western Blots / Immunoblotting

In western blotting (or immunoblotting), sample proteins are first separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE). This provides information about the molecular weight and the potential existence of different isoforms of the proteins under consideration (Figure 15-5).

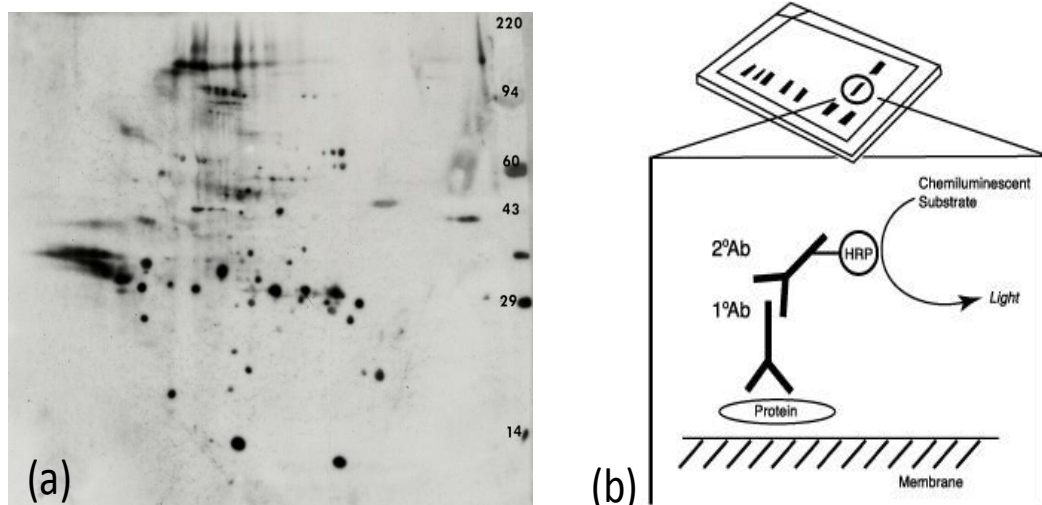


Figure 15.5. (a) Protein separation by 2-dimensional gel electrophoresis prior to blotting. (b) Antibody detection system following protein transfer to membrane.

These proteins are subsequently immobilised typically onto polyvinylidene difluoride (PVDF) or nitrocellulose membranes before identification using labelled monoclonal or polyclonal antibodies. The limitations of this technique are often demonstrated at the transfer / immobilisation step whereby a poor quality transfer will result in problems with the immunoblotting process. Similarly, inadequate assay optimisation may result in membranes with a high background or low signal to noise ratio.

Similar to the PCR arrays discussed previously, commercially available protein arrays are now readily available. The most common protein microarrays use protein specific antibody pre-spotted onto a support medium. Proteins of interest may be then detected directly from cell lysate solutions. These arrays may also be custom made to investigate specific biological pathways or specific disease states.

15.4.4. Proteomics

Analogous to the genome, the proteome is the entire complement of proteins including the modifications made to a particular set of proteins, produced by an organism or system. [37] Identification of this set of proteins is no easy task given that the 35,000 genes in the human genome can code for at least ten times as many proteins. Proteomics i.e. the comparison of proteomes between different systems therefore often relies on high throughput techniques using mass spectrometry to sequence peptide fragments prepared from proteins isolated from the experimental system(s). The peptides identified in this way may then be compared to databases of all possible proteins generated *in silico* from the human genome database.

Recently, proteomic analysis has been used to determine the nature of the gingival tissue exudate (gingival crevicular fluid) around teeth [38] and hence future utilisation of proteomics in implant dentistry may be focused on identifying diagnostic factors associated with peri-implantitis by characterizing the proteins in the peri-implant tissue exudate during health and disease.

Conclusion

Following implant placement, a multitude of processes within an array of cell types are activated. By way of a broad summary, these processes can include the mechanical and biochemical activation of cellular receptors leading to the modulation and regulation of intracellular signalling pathways. The participants of these pathways subsequently transduce the signal to the nucleus thereby affecting specific gene expression leading to specific protein(s) production, cellular proliferation, differentiation and ultimately osseointegration. Similarly, disease processes that influence the success of implants elicit a cascade of events that involve mRNA expression, induction of signalling pathways and ultimately protein production.

The analysis of gene expression in particular, through the development of molecular biology technologies, has provided an insight into the fundamental cellular and molecular mechanisms that result in osseointegration. The molecular biological techniques introduced

above can provide us with the tools necessary to further elucidate the underlying mechanisms involved in osseointegration, as well as the disease processes that can lead to implant related pathology. This understanding may then be translated into clinical practice by providing leads for further implant development based on a sound understanding of the osseointegration process at the molecular level.

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Chapter XVI

Bioengineering Applied to Oral Implantology. Biomechanical Studies

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16.1. Introduction

16.1.1. Forces and Stresses

This chapter concerns itself with forces, stresses and strains as they are generated during a patient's functional or parafunctional activity. Very schematically, when the patients start closing their mouths, the peri-oral muscles will shorten and, as long as the mandible freely moves in its envelope of motion, no clinically relevant force is generated. The situation abruptly changes when the teeth make contact. At this time, the contraction of the muscles forces two opposing teeth together. While the forces thus applied are generated by the muscles, they are transferred to the occlusal surfaces via the maxillary and mandibular bones, the periodontal ligament, the dentinal core of the teeth and, finally, the external layer of enamel.

Since the tissues are all linked together, they are said to form a *mechanical continuum*. The above describes the chain of events in natural teeth but would equally apply to osseointegrated implants carrying a restoration. In the latter instance, the continuum comprises the osseous structures, the various components of the implant pillar and the fixed or removable prostheses replacing the teeth.

The forces generated by the muscles will distribute themselves inside the various structures of the continuum. When the structures are of plain shape (such as a cylinder) and homogeneous composition, they will distribute themselves evenly across the medium. When the structure's geometry is more intricate (such as the screw-on component of an implant for instance), the forces' distribution may become quite complex and often concentrate in some areas.

In these zones, the nominal load may be multiplied by several times. It thus makes sense to describe the force situation not relative to the external load applied to the structure but in terms of the total force that develops on a specific surface area. The latter is called stress and written as Force / area [N/m^2], which yields Pa (Pascals) or MPa (Megapascals) if the area is in mm^2 . As elementary examples of such computations, consider Figure 16–1A and 16–1B.

The stress concentrating effect of more intricate geometries is depicted in Figure 16–2 in which a notch has been machined in the midportion of a cylinder (Figure 16–2A). Note how the force flow lines, which are evenly distributed in the top and bottom parts of the cylinder, now concentrate around the notch tip in Figure 16–2B, in effect locally magnifying the nominal stress by several times. The concentrating effect is a function of the notch's depth and the radius at its tip (Figure 16–2C), both of which determine the *stress concentration factor* (Figure 16–2D).

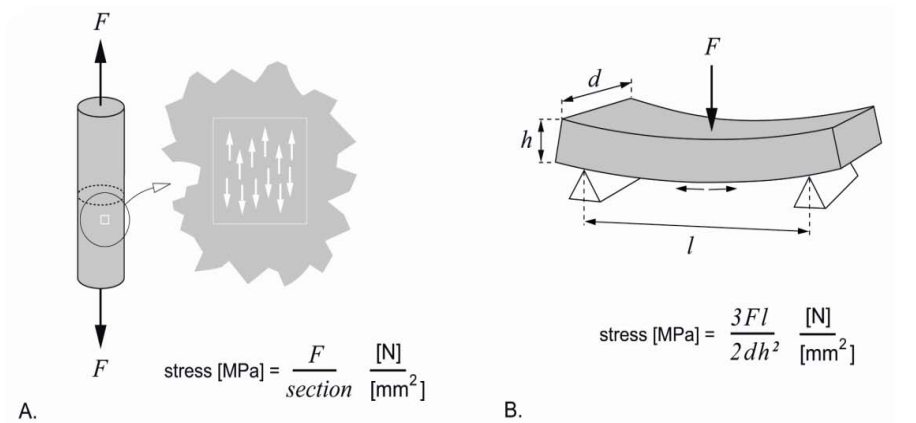


Figure 16.1. A, B. Compute stress A. In a cylinder placed in tension (or compression), stress is the applied force divided by the surface. B. On a bending beam, the stress on the outer tensile layer is calculated using the parameters shown.

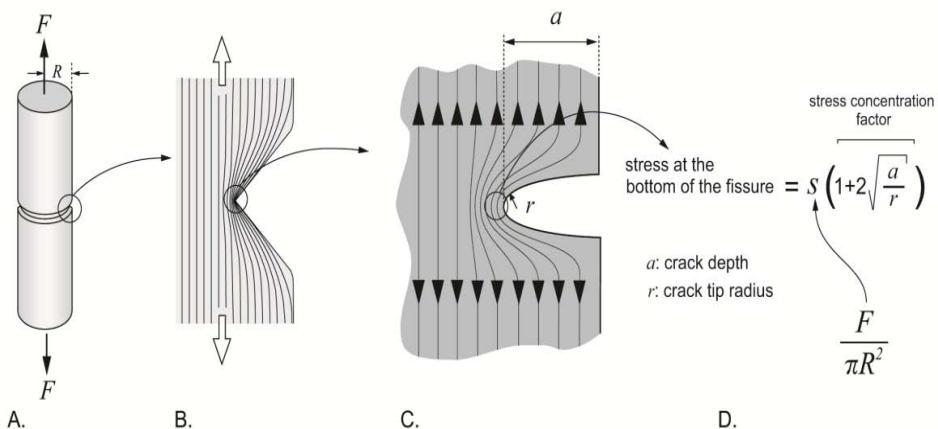


Figure 16.2. A-D. Stress concentration A. A grooved rod is subjected to pulling. B. The stress lines concentrate around the groove tip. C,D. The stress concentration factor is a function of the crack depth and the radius of the notch's bottom.

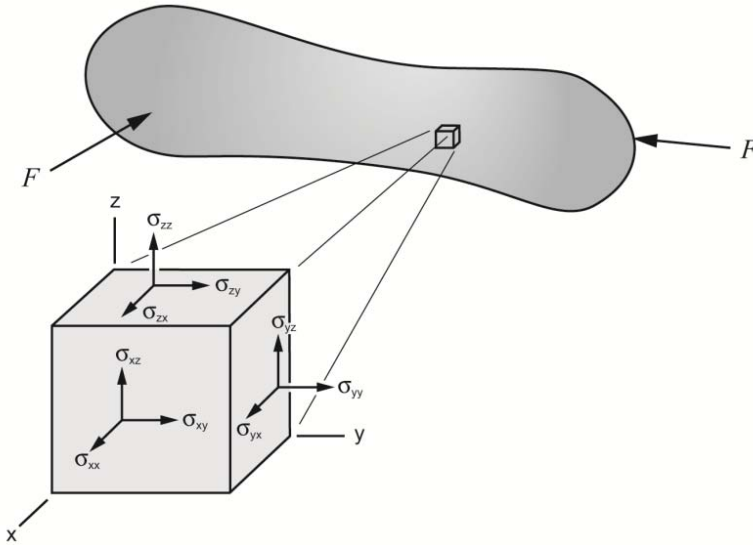


Figure 16.3. Stress components A body (of any shape) is subjected to a force system (F). The forces within the body may then be pictured using an infinitesimal cube of material. Each side of these cubes is subjected to three orthogonal force vectors (two opposing sides of the cube resolve into one). These are the nine components of the stress tensor.

In the zone surrounding the notch tip, the nominal stress (as taken from Figure 16–2A) is now multiplied by the stress concentration factor. Being subjected to much higher levels of stress, the zones where stresses concentrate carry an increased risk of breakage. Knowing that such concentrations are solely due to the geometry of the component under investigation (the material plays no role in this affair), they must be located and, if possible, alleviated to diminish the concentrating effect. For the sake of completeness though, let us mention a special load case that arises in zones of bearing contact between two structures. Whenever one structure is much stiffer than the other and possibly indents the adjacent part, it will locally deform the softer material and also generate local stress concentrations.

In elementary situations, stresses can be computed using basic mathematical formulae, so-called *closed formulations*. Typically though, these apply to tensile loads only (compression is considered a "negative" tension). The two other loading modes, that is, shear and torsion, significantly increase the complexity of the mathematical treatment.

This combination of outer geometry and externally applied force systems creates patterns of stress distributions inside the body (a "body" is a volume of homogenous or inhomogeneous material that can take any conceivable shape). These are referred to as *stress fields*. When they are computed for each infinitesimal volume of the body, the stress fields resolve into the *stress states* of each location (Figure 16–3).

The nine stress elements of Figure 16–3 are typically organized into a matrix of the form

$$\begin{bmatrix} \sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\ \sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\ \sigma_{zx} & \sigma_{zy} & \sigma_{zz} \end{bmatrix}$$

which is called the *stress tensor* (or stress matrix). In effect, such stress tensors should be viewed as if each of the three planes of space were subjected to its own stress system. The latter explains the notation. The first index (i.e., x, y or z) is the plane along which the vector acts. The second index is the stress component relative to that plane.

With respect to Figure 16–3, it can be demonstrated that the stress vectors acting on each of the three planes of space may themselves resolve into (i.e., "expressed as") three mutually orthogonal stress components, that is, one that is normal to the plane of interest (denoted as sigma) and two shear components (denoted as tau) thus resulting in the diagram shown in Figure 14–4.

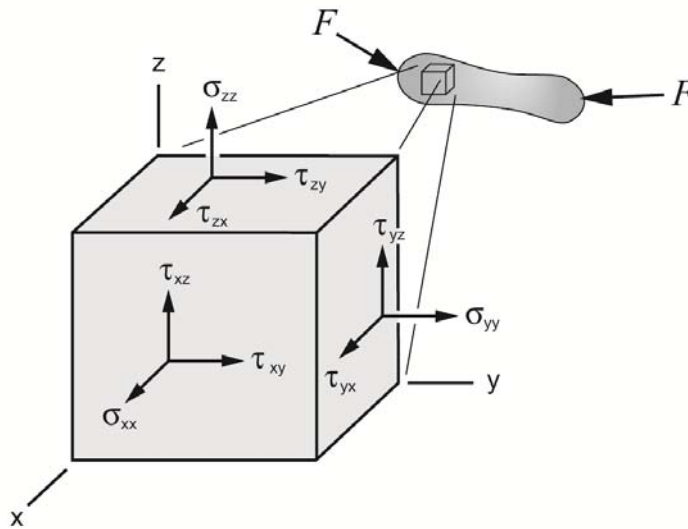


Figure 16.4. Shear stress components The stresses normal to the planes are purely tensile/compressive (denoted as σ). The two other components (i.e., the stresses parallel to the plane) are in shear (denoted as τ).

The stress matrix may thus be reformulated as the so-called *Cauchy stress tensor*, that is

$$\begin{bmatrix} \sigma_x & \tau_{xy} & \tau_{xz} \\ \tau_{yx} & \sigma_y & \tau_{yz} \\ \tau_{zx} & \tau_{zy} & \sigma_z \end{bmatrix}$$

To proceed to the last step of this demonstration, we need to simplify matters and assume that the stress component in the z direction is zero. The body is now stressed along the x and y axes only – a condition referred to as *plane stress*. Such a plane stress situation is diagrammed in Figure 16–5. Note that both axes of the plane are subjected to one component that acts normal to the face (σ) and one that acts in shear (τ). The point of this development is that the body may be rotated inside this force system and, at one specific angle (θ_p) (relative to the x-y coordinate system), the shear stresses reduces to zero. At this time, only tensile and compressive stresses are active.

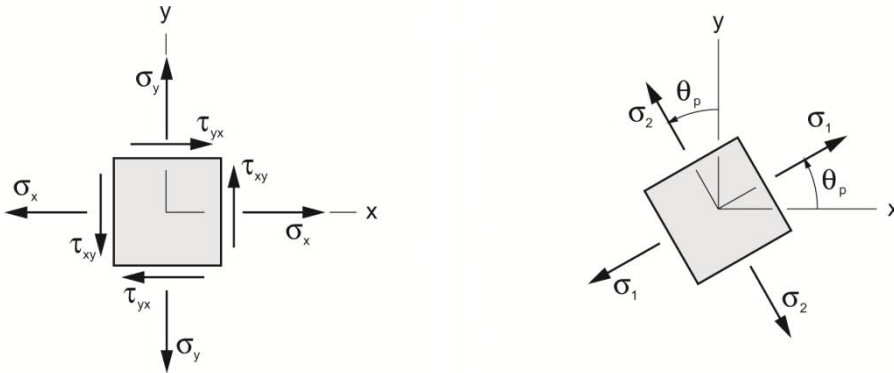


Figure 16.5. Principal stresses This example pertains to plane stress (i.e., in two dimensions, σ_x , σ_y , τ_{xy} , τ_{yx} are the stress components acting on the square. By rotating the square by the angle θ_p (Greek letter ρ), the shear stresses are eliminated, and the system reduces to two orthogonal tensile/compressive stresses: the principal stresses.

These are referred to as *principal stresses*. Evidently, the same holds true for three-dimensional situations, but the problem somewhat increases in complexity.

Complex body geometries and force systems will generate internal stresses that escape basic mathematical formulations and thus must be approached using more elaborate techniques. We will describe two of these below, that is, photoelasticity and the finite element method.

16.1.2. Strains

Any body onto which a force is applied will deform. This deformation is called strain. Strains may be tensile (elongation) and compressive (contraction). To distinguish between them, tensile strains are positive, and compressive strains are negative (beware: some authors do not follow this rule). For a cylindrical rod subjected to a tensile force, strain is defined as the ratio between the rod's length after it has been pulled and its length at rest: $l_o + dl$, where l_o is the original length (in millimeters) and $l_o + dl$ the length after pulling. When the applied force is converted into stress, the body deforms following a well-defined course that is typically plotted as a stress-strain diagram (actually a strain-stress diagram, since strain is the independent variable) (Figure 16–6). In engineering, σ (sigma) is the stress and ϵ (epsilon) is the strain.

A host of parameters can be derived from stress-strain diagrams – all of which characterize a material's reaction to load application, in particular regarding deformation and strength. In the context of the present discussion, we will make use of the linear portion of the plot, i.e., that in which it may be strained and revert back to its original shape. This portion is called the elastic (or “Hookian”) domain and indicates the strain range that induces no (apparent) permanent damage to the material. The slope of this line segment is called the *modulus of elasticity* (or *Young's modulus*).

The second parameter of use is *Poisson's ratio* (or modulus), that is the relationship between the constriction (in diameter) and the elongation (in length) of the specimen (typically a cylinder) when subjected to a tensile force. Poisson's ratio may take any value

between zero and 0.49. The Greek symbol for Poisson's ratio is ν (nu). Its components are explained in Figure 16–7.

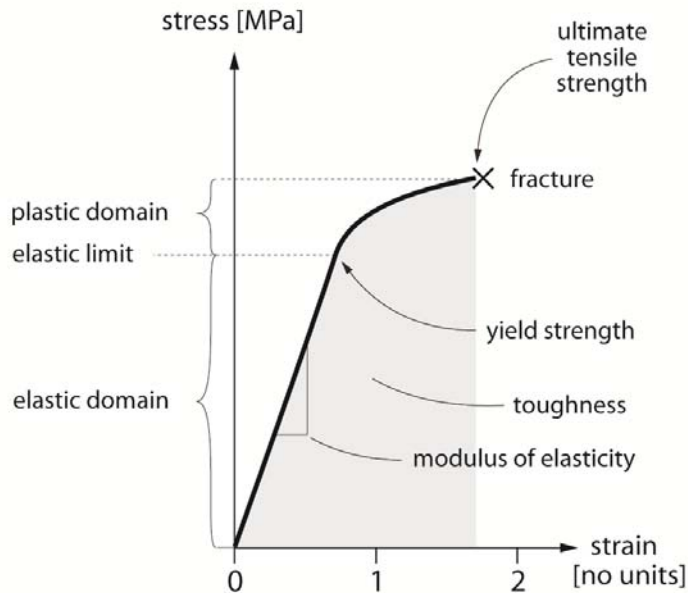
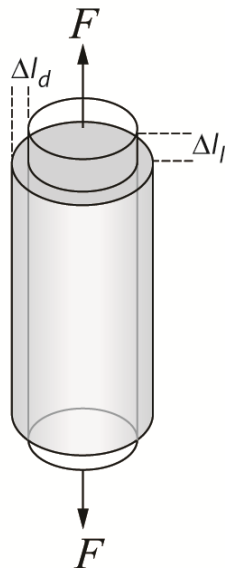


Figure 16.6. Stress-strain diagram The shaded area is called toughness – a parameter that characterizes a material's strength. It may be viewed as the amount of energy absorbed by the material before breakage.

We will make use of the stress tensor, the modulus of elasticity and Poisson's ratio when we discuss the principles of strain gauges and finite element analyses.



$$e_t = \frac{\Delta l_d}{\text{initial diameter}}$$

$$e_l = \frac{\Delta l_l}{\text{initial length}}$$

Poisson's ratio (ν):

$$\nu = - \frac{e_t}{e_l}$$

Figure 16.7. Poisson's ratio Poisson's ratio is the ratio between the constriction (in diameter) and the elongation (in length).

16.1.3. Assessing Stresses and Strains

In spite of it being a fundamental notion in mechanics, stress cannot be measured directly as it must be derived from strain. Strain is a physical entity that is amenable to measurement by the way of strain gauges, that is, sensors designed to measure fine scale deformations on the surface of a component (other methods exist, but strain gauges are the most commonly used).

The typical load cell therefore does not measure a force. It measures the deformation in reaction to the force. Being tested in elongation (or compression), the material that makes up the load cell must respond to the following specifications [1]:

- **Linearity.** The relationship between the applied force and the material's deformation is linear. This in turn will yield a linear relation between the deformation (translating into strain) and stress (as calculated from strain multiplied by the modulus of elasticity).
- **Elasticity.** The deformation due to external loading is completely and instantaneously reversible upon removal of the applied force.
- **Continuity.** The distribution of matter within the material is continuous on all size scales. There are no flaws or voids.
- **Isotropy.** The material is homogeneous throughout. This translates into a material that presents the same mechanical properties in all three directions of space.

Strain gauges and their laboratory applications in the form of material testing machines are the only devices by which mechanical entities can actually be measured. Stress determinations based on photoelasticity and, more importantly, on finite element analyses are only mathematical constructs.

16.2. Strain Gauges

16.2.1. Introduction

Strain gauges (or *gages*) belong to a set of experimental techniques that assess strains arising at the surface of a structure. These techniques therefore, will not provide information regarding stresses in the deeper layers and require that the surfaces under scrutiny be readily accessible to the experimentalist.

Three techniques belong to this category of measurements. The first is a purely graphical method referred to as “grid technique.” It consists of scribing a series of squares onto a surface. These squares can be drawn with fine ink or photo-etched into the surface. When the component is subjected to loading, the grid deforms – a deformation that is analyzed using a micrometer microscope.

A second technique consists of utilizing brittle lacquers that will develop cracks when the structure is loaded. This technique, referred to as *brittle coatings*, is briefly presented below.

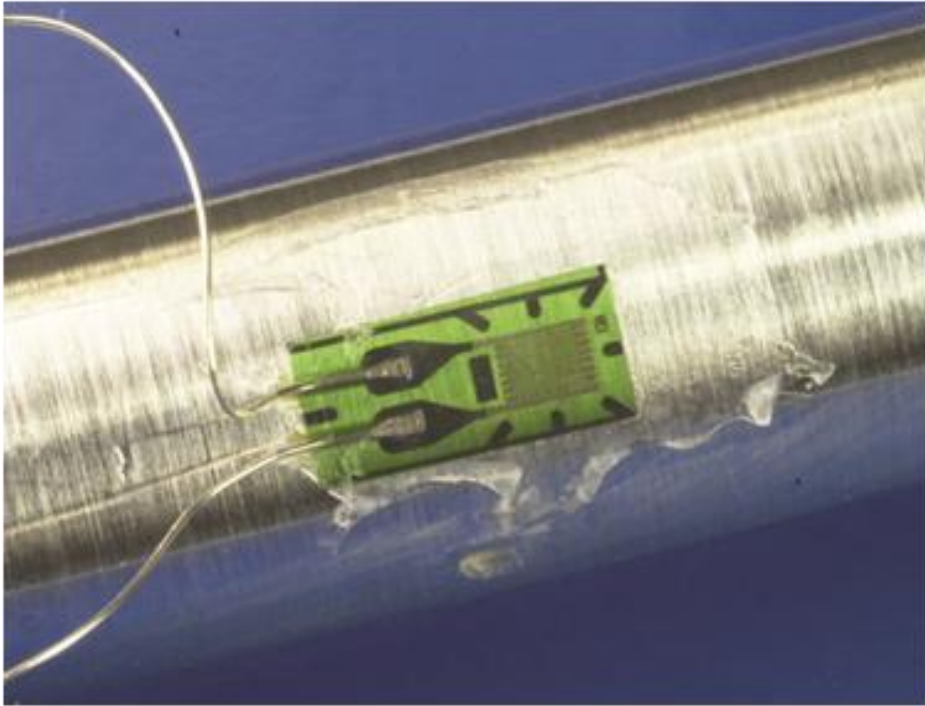


Figure 16.8. Strain gauge. Strain gauge bonded to the surface of a machined rod. Picture courtesy of Department of materials science and metallurgy, U. of Cambridge, UK [2].

The third method is the object of this section and consists of utilizing strain gauges. These devices present themselves as assemblages of small wires and are a quintessential tool in the measurement of deformations that may be either tensile (translating in elongation) or compressive (translating into shortening). Strain gauges can thus be used to measure expansion as well as contraction.

Typically, strain gauges are bonded to the surface of an object (Figure 16–8). Under load, both the substrate component and the gauge deform alongside, and the strain registered by the gauge thus closely duplicates the strain on the underlying surface.

The strain range that is picked up by a gauge may be exceedingly small so that strains in the 10^{-3} to 10^{-4} range are not uncommon. In these instances, it is customary to switch to a somewhat different scale called *microstrain* ($\mu\epsilon$). One microstrain is a deformation of 10^{-6} . 10^{-4} would be $100\mu\epsilon$, 10^{-3} $1000\mu\epsilon$ and so on.

Brittle Coatings

As a fully analogue (i.e., non-digital) method that exemplifies the use of strain gauges, brittle coating testing will be briefly presented and discussed. Brittle coating is a non-destructive technique that provides the experimentalist with a visual appreciation of the strain response of the material onto which it is applied. It consists of applying several coats of a specially formulated lacquer onto the specimen under scrutiny. When the lacquer has dried, the specimen is loaded, and the coating will crack in zones of supracritical strain. (“Supracritical” means that the lacquer starts developing cracks when strained beyond a specific (i.e., “critical”) value).

By design, the technique applies to surface strains in tensile mode. Typically, after coating application, loads that simulate service conditions are applied to the structure, which is then inspected for the opening of cracks. The method thus expediently indicates areas at risk.

Principle

The brittle coating fractures in response to the strain generated in the underlying surface. Besides the mere detection of strains, the coating also specifies the direction of the principal stress as the cracks develop perpendicularly to the resultant of the principal strains (Figure 16–9).

Moreover, it will also indicate its order of magnitude. This is achieved by utilizing lacquers of different critical strains (relevant figures range from 10^{-4} to 10^{-3}). In due consideration of the material's modulus of elasticity, the experimentalist gauges the lacquer's critical strain so that it falls within the range of functional deformations. The quantitative data thus gathered are reproducible within $\pm 10\%$. Also note that cracking should occur at a level within the structure's elastic range. Testing in the plastic range (i.e., beyond the yield stress) is normally not part of the test. While major cracks are plainly visible, more subtle fissures can be detected and photographed using special electrostatically charged powders that build up at crack interfaces [3].

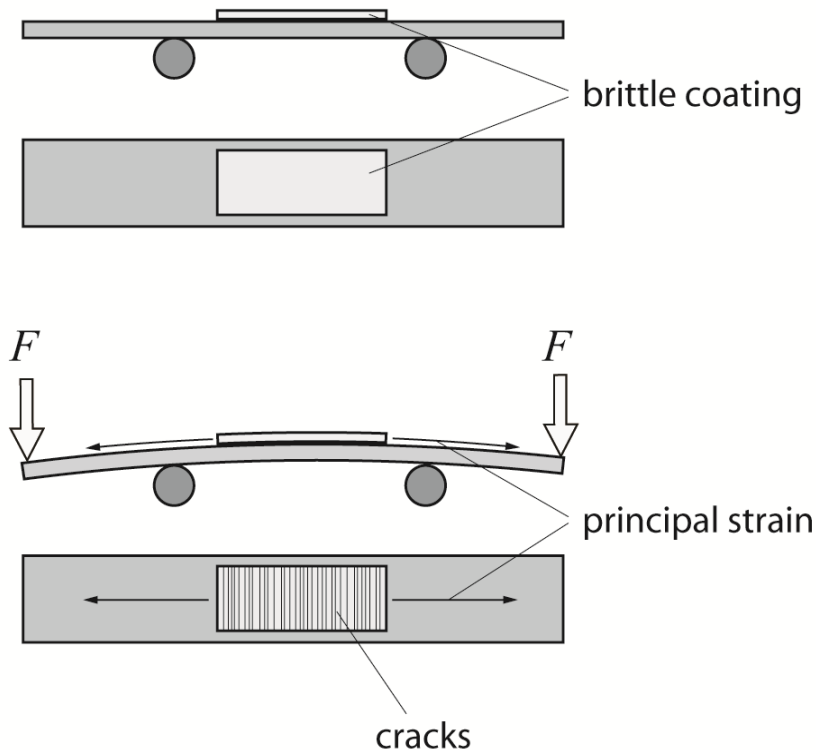


Figure 16.9. Principle of brittle coating The brittle coating cracks perpendicular to the direction of the principal strains. Adapted from School of mechanical and mining engineering, U. of Queensland, Australia.

Advantages/Disadvantages

The brittle coating method is a technique that expediently provides an overall picture of the stress distribution on a given surface. As such, it will also indicate zones of stress concentrations. On the downside, the technique requires a 100% accessibility of the surface under scrutiny, it does not permit the conversion of the observations to an electrical signal and it is an essentially qualitative method that does not allow actual measurements.

16.2.2. Principles of Strain Gauges

Basically, a strain gauge is a detector that converts small-scale mechanical motions into electric signals. The strain gauge is tightly bonded to a substrate object so that the sensing element may elongate or contract according to the strain borne by the object to be measured. The elementary strain gauge consists of an interconnected wire lattice through which an electrical current flows. The gauge is designed to register strains in the direction parallel to the lattice, while transverse strains will only marginally affect the gauge's response. The deformation experienced by the device translates into a change in electrical resistance that is proportional to the strain applied. By registering and interpreting these changes, the experimentalist is able to deduct the underlying strain (Figure 16–10).

Strain gauges working under the capacitive (as in a capacitor) and others under the inductance (as in a coil) principle also exist. Problematically, these devices are sensitive to vibration, they are intricate in their mounting requirements and their electric circuitry is complex. Therefore, it is the variation in electric resistance that is mostly used.

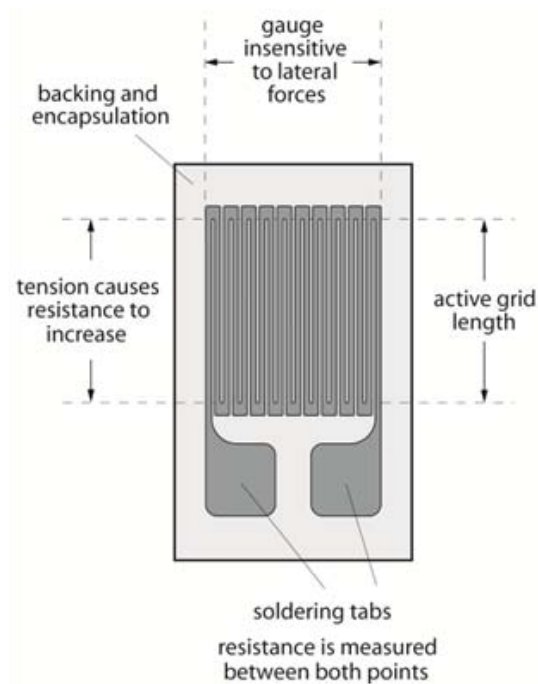


Figure 16.10. Strain gauge principle The grid reacts to changes in length. Tension causes the resistance to increase. Conversely, when compressed, the resistance measured between both soldering tabs decreases.

When the conductor is stretched within its elastic range (i.e., so that it does not break or permanently deform), it will become narrower and longer. These changes will increase its electrical resistance. Conversely, when a conductor is compressed, it will somewhat increase in cross-section and shorten so that its overall electrical resistance is decreased.

Strain Gauge Types

The three classical electric conductors inside a strain gauge are the following (i) wire, (ii) foil or (iii) semiconductor. We discuss these three types in Section 16.2.4. More recently developed varieties comprise (iv) thin film strain gauges, (v) diffused semiconductor gauges and a technology known as fiber Bragg grating. In the thin film method, the gauge is intimately bonded to the underlying structure using vacuum deposition or sputtering technologies (and not bonded by hand using glue as in the traditional method). To this effect, an electric insulation (usually a ceramic) is deposited first, and the strain gauge is affixed secondarily.

This vastly improves the stability of these installations and significantly reduces their drift. In the diffused semiconductor method, the strain gauge is layered using photolithography masking techniques and solid-state diffusion of boron. Eliminating bonding agents largely eliminates errors due to creep and hysteresis. This technology is often integrated into pressures sensors. Fiber Bragg grating is an optical method in which the refractive index of a light-transmitting fiber is altered at periodic intervals. When the fiber is stretched, this alters the incoming light beam in a manner that can be interpreted in terms of strain. This technology, however, is used for much larger measurements than intraoral dimensions.

16.2.3. Background theory [4,5]

The resistance (R) of an electric conductor obeys the following relation:

$$R = \rho \frac{l}{a}$$

where ρ is the resistivity of the wire (i.e., the inherent resistance of the material), l is the length and a is the conductor's section. This equation may be rewritten in terms of natural logarithms, and we thus obtain:

$$\ln(R) = \ln(l) + \ln(\rho) - \ln(a) \quad (\text{eq.2})$$

Eq. 2 may now be differentiated using the following relation:

$$\frac{d}{dx}(\ln(x)) = \frac{1}{x} \Rightarrow d(\ln(x)) = \frac{dx}{x}$$

and thus rewrites to:

$$\frac{dR}{R} = \frac{d\rho}{\rho} + \frac{dl}{l} - \frac{da}{a} \quad (\text{eq.3})$$

For a cylindrical wire, $a = \pi r^2$ (r = radius). Therefore, taking the same steps for πr^2 as above for a (i.e., natural logarithm followed by differentiation) yields $2 \frac{dr}{r}$, which we may now substitute for the area term in eq.3:

$$\frac{dR}{R} = \frac{d\rho}{\rho} + \frac{dl}{l} - 2 \frac{dr}{r} \quad (\text{eq.4})$$

To pursue, we need to "inflate" eq. 4 to:

$$\frac{dR}{R} = \frac{d\rho}{\rho} + \frac{dl}{l} \left[1 - 2 \frac{dr/r}{dl/l} \right] \quad (\text{eq.5})$$

Using this little trick, we may now substitute $\frac{dl}{l}$ for the elongation ratio (i.e., strain) in the axial direction (ε) and

$\frac{dr/r}{dl/l}$ for the wire's Poisson ratio (ν):

$$\frac{dR}{R} = \frac{d\rho}{\rho} + \varepsilon (1 + 2\nu) \quad (\text{eq.6})$$

One important parameter is included in eq. 6. It is called the *gauge factor* (F) and relates the variation in overall resistance to the strain of the wire. Therefore, we may write

$$F = \frac{dR/R}{\varepsilon} \rightarrow F = \frac{dR/R}{\varepsilon}$$

Note that the gauge factor is also referred to as the gauge's *sensitivity*. In these instances, it may be denoted as K . The components of the gauge factor may be substitutes into eq.6 to obtain:

$$F = 1 + 2\nu + \frac{1}{\varepsilon} \frac{d\rho}{\rho} \quad (\text{eq.7})$$

For the computation of local strains in terms of the gauge factor, the total resistance of the wire and the change in resistance, we would thus utilize a more restricted relation:

$$\varepsilon = \frac{1}{F} \frac{\Delta R}{R} \rightarrow \varepsilon = \frac{1}{F} \frac{\Delta R}{R} \quad (\text{eq.8})$$

Finally, assuming that the resistivity of the material is not strain-dependent, eq.7 simplifies to

$$F = 1 + 2\nu$$

Numerical Example

From eq.8, we know that the strain (i.e., the parameter we want to measure) is defined as:

$$\frac{\Delta L}{L} = \frac{1}{F} \frac{\Delta R}{R} \rightarrow \frac{\Delta L}{L} = \frac{1}{F} \frac{\Delta R}{R} \quad (\text{eq.9})$$

with R = the initial resistance (in ohm $[\Omega]$), ΔR = change in resistance, L = the initial length [mm] and ΔL the change in length.

For this example, we will assume an unloaded strain gauge resistance of 120Ω and a gauge factor of 2 (both are typical values). Experimentally, we measure an increase in the gauge's resistance of 0.24Ω . Substituting, we obtain

$$\varepsilon = \frac{\Delta L}{L} \rightarrow \varepsilon = \frac{\Delta L}{L} = \frac{0.24}{2 \cdot 120} = 0.001 \text{ (or } 1000 \mu\varepsilon)$$

In practice, it is nearly impossible to measure changes in resistance in these orders of magnitude (at least not with a conventional ohmmeter). Therefore, one uses a dedicated amplifying circuitry called a Wheatstone bridge, which will be described in Sect.16.2.5.

16.2.4. Gauge Types

Strain gauges are selected in consideration of several factors, most importantly the gauge factor. Problematically, all strain gauge materials are also sensitive to variations in their environment and tend to change their resistance value as they age (the latter is called drift). For tests of short duration in a constant environment, these aspects need not be a concern. However, when tests are conducted over prolonged periods and the gauge is taken in and out of the oral cavity, appropriate compensations must be included into the electric circuitry.

As a general statement, the ideal gauge material

- responds to strain with large variations in resistance,
- maintains its gauge factor constant over a range of temperatures,
- is of low modulus of elasticity,
- can be easily processed to a bondable strain gauge.

An overview of typical alloys and semi-conductors is provided in Table 16–1.

Table 16.1. Electric specifications of commonly used strain gauge materials Note that typical values for F (gauge factor) lie between 2 and 4. The temperature coefficient of resistance (α) denotes the variation in resistance per degree of temperature change

Denomination	Composition	Gauge factor (sensitivity)	Resistivity [$\mu\Omega$ cm @ 20°C]	Temperature coefficient of resistance [$^{\circ}\text{C}^{-1} 10^{-6}$]
Constantan	55%Cu, 45%Ni	2.1	49	-40 - +40
Nichrome V	80%Ni, 20% Cr	2	108	40 – 100
Karma	74%Ni, 20%Cr, 3%Al, 3%Fe	2	125	± 20
Manganin	84%Cu, 12%Mn, 4%Ni	0.47	± 15	0 – 40
Isoelastic	55.5%Fe, 36%Ni, 8%Cr, 0.5%Mo	3.6	110	450
Platinum alloys	5%Ir, 8%W, Pt(bal)	4 - 5.1	24	3'800
Semiconductors	Si-, Ge-based	-125 - +175	4'600 - 10^6	-60'000

Table was re-arranged.

Wire Gauges

As indicated in their name, these gauges are made of thin wire filaments approximately 0.025mm in diameter. They were developed in the 1930s to measure vibratory strains in high performance propeller blades. The first gauges were wound with fine copper wire on a thin paper tube. These gauges had the advantage of smaller grid dimensions but also presented higher levels of creep. The typical contemporary wire gauge, though, is of the flat wire type in which the filament is zigzagged between two pieces of paper.

Problematically, wire gauges are not amenable to mass production and still must be largely produced by hand. They present some technical intricacies and are more expensive than foil types. Therefore, they are not widely used.

Foil Gauges

In 1952, the English firm Saunders-Roe were seeking improvements in the performance of the bonded wire gauges to enable their use in more demanding environments. At that time, printed circuits were emerging, and Saunders-Roe developed the idea of fabricating a strain gauge by etching the pattern for the gauge from a thin foil. These foil gauges had some distinct advantages, most notably a reduction in size and production costs. This allowed a much more extensive use of electric resistance strain gauges, and foil gauges are the most common type in use today⁶ (Figure 16–11).

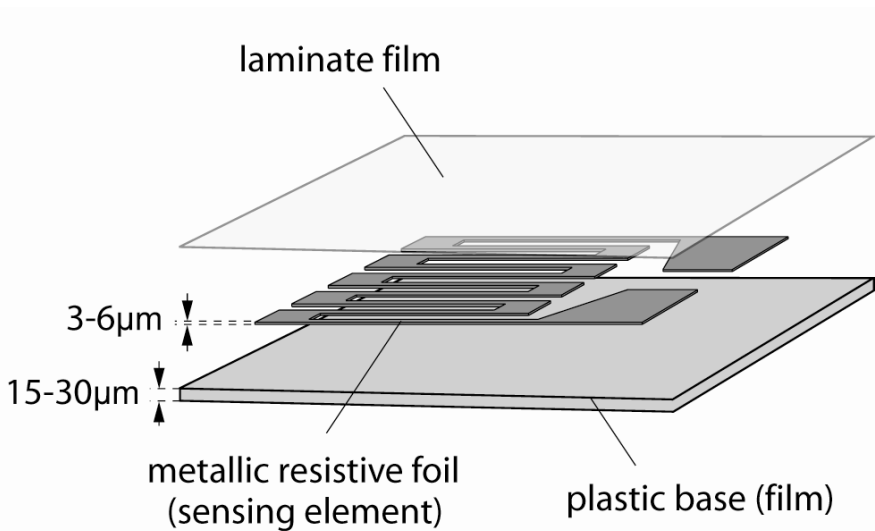


Figure 16.11. Diagrammatic view of a foil gauge The typical foil gauge is structured into a grid-shaped sensing element of thin metallic resistive foil (3-6µm in thickness), which is layered on a plastic base (15-30µm) and is covered with a thin laminate film.

In the typical foil gauge, the electric conductor is made of a 0.003 to 0.006mm thick resistive metal film. The gauge backing is an epoxy resin in the 0.015 - 0.03mm range, which provides the electrical insulation with the component to be tested. These gauges are available in a wide choice of shapes, sizes and arrangements to suit a variety of applications (we present some of these in Sect.16.2.6).

While early foil gauges were essentially printed circuits, contemporary foils have become highly sophisticated devices. The foil is a small and thin film that is vapor-deposited on a slim plastic surface. Advanced technologies enable strain gauges manufacturers to decrease their overall size into the millimeter range.

Semiconductor Gauges

Semiconductor strain gauges were developed for the automotive industry in the 1970s. Unlike metal gauges, they are based on the piezoresistive effect of silicon or germanium. *Piezoresistance* is the change in resistance of a semiconductor under applied mechanical stress. In contrast to its “piezoelectric” sibling, the “piezoresistive” effect only causes a change in electric resistance; it does not produce an electric potential.

The typical semiconductor gauge is a wafer of about 0.25mm thickness with the resistance element diffused into a substrate of silicon (Figure 16–12). These gauges present much larger gauge factors than metal gauges, which were at one time thought to herald the downfall of their metallic counterpart. Problematically, semiconductor gauges are also much more sensitive to temperature variations, and they tend to drift. Further, the resistance-to-strain relationship is nonlinear, varying by 10-20% from a straight-line equation. Therefore, semiconductor gauges are not general-purpose devices, and their use is restricted to specific applications.

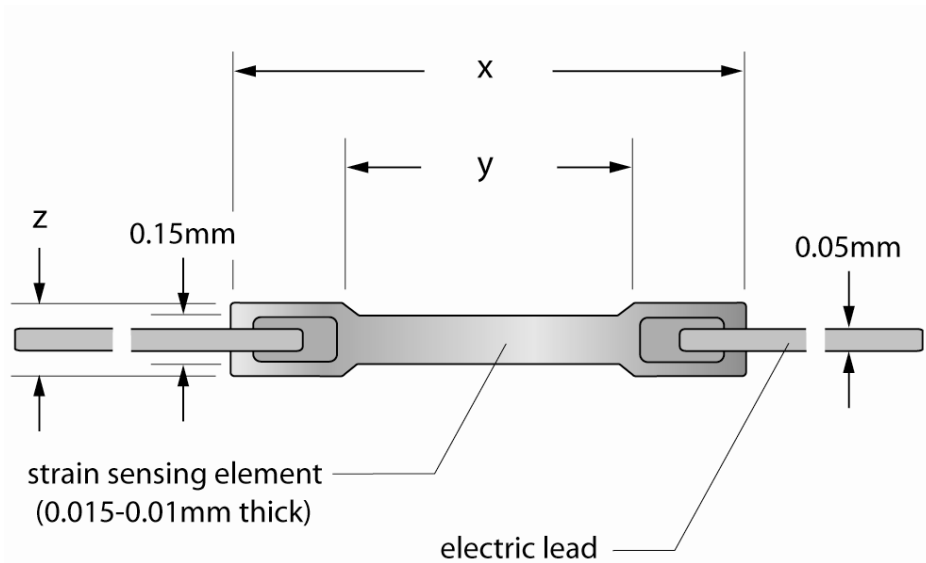


Figure 16.12. Diagrammatic view of a semiconductor gauge (bar type). x ranges from 0.68 to 6.35mm, y from 0.33 to 5.7mm and z from 0.20 to 0.23mm. Adapted from ATI Corp.

16.2.5. Implementation

Strain Gauge Placement

Some strain gauges, such as in pressure vessels or load cells, are incorporated into the substrate structure. The all-purpose strain gauge though is affixed to the substrate using glue. The following adhesives are available:

- Cyanoacrylate cement,
- Epoxies,
- Ceramic cement,
- Cellulose nitrate cement.

The type of glue depends on the duration of the test. For short-term measurements (up to some weeks) cyanoacrylate glue is appropriate; for long-lasting installation, epoxy glue is required. Usually epoxy glue requires high temperature curing (at about 80-100°C) but will ensure a strong bond. Ceramic cements are used in high-temperature environments. Cellulose-nitrate cements are suitable for paper-backed strain gauges and thus restricted to dry conditions. Independent of its application, the ideal adhesive (i) may be applied in thin coats, (ii) is suitable for a range of temperatures and (iii) behaves as an insulator.

Bonding strain gauges to test specimens may appear straightforward, but it is not. Gauge bonding is a craft in its own right, as flawless placement is absolutely essential for accurate and stable strain measurements. Hence, the reader is advised to obtain counseling and to practice under supervision before engaging in tests involving bonded strain gauges. Most manufacturers provide technical notes and handling instructions.

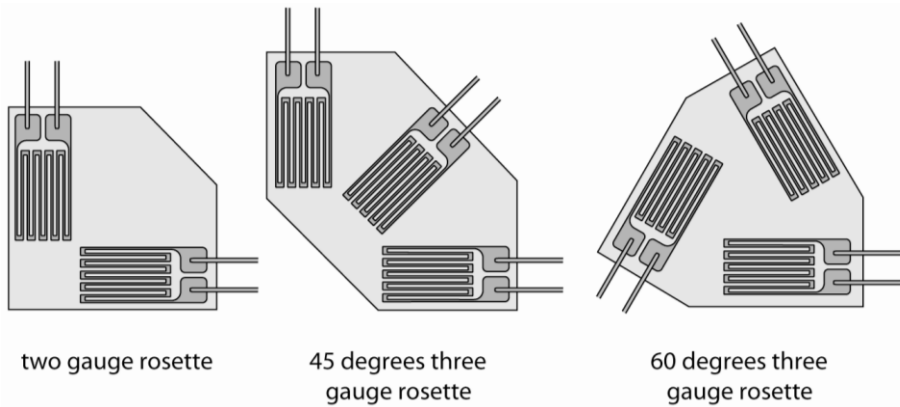


Figure 16.13. Rosette strain gauges. To determine the direction of the resulting principal strain, two or three gauges are arranged on a single pad. The active axes of the gauges are oriented at 45, 60 or 90 degrees of each other.

As a first step, the surface onto which the strain gauge is to be bonded must be smoothed with fine sand paper (e.g., with 320 grit first, followed by 400 or finer grit) and degreased with a variety of solvents (alcohol, acetone, or some other degreasing agent). Then the strain gauge must be glued immediately to avoid oxidation or pollution of the prepared area. If these preliminary steps are not followed, the strain gauge's binding to the surface will be unreliable, and unpredictable measurements will result.

In many instances, the orientation of the gauge on the workpiece will derive from the direction of the externally applied load. In these instances, the active axis (Figure 16–10) of the gauge will be placed parallel to the resulting principal strain. In some instances though, the stress distribution in the structure may be complex, thereby requiring that several gauges be placed in a variety of directions. To this end, the industry provides sets of two or three identical gauges that are arranged on a single base with their axes oriented at 45, 60 or 90 degrees of each other. Such *rosette strain gauges* are depicted in Figure 16–13.

Ancillary Electric Circuitry

The sensing element of the strain gauge converts variations in length to corresponding changes in electric resistance (eq.9). However, the strains under scrutiny are nearly infinitesimal. Therefore, the induced changes in resistance are extremely small, and it is almost impossible to assess this minute variation directly. The problem has been circumvented by "extracting" the gauge's resistance using a special electrical circuitry called a *Wheatstone bridge*.

Wheatstone Bridge

The Wheatstone bridge is an electric circuit that lends itself to the measurement of small changes in resistance by its clever arrangement of resistors and voltage measurements. Its most elementary form is shown in Figure 16–14.

The Wheatstone bridge comprises four resistors, arranged as in Figure 16–14A,B. The circuit is powered by a DC (i.e., direct current) source voltage (also referred to as "excitation voltage") and also features a voltmeter (i.e., to measure the output voltage) that will indicate any imbalance in the resistors.

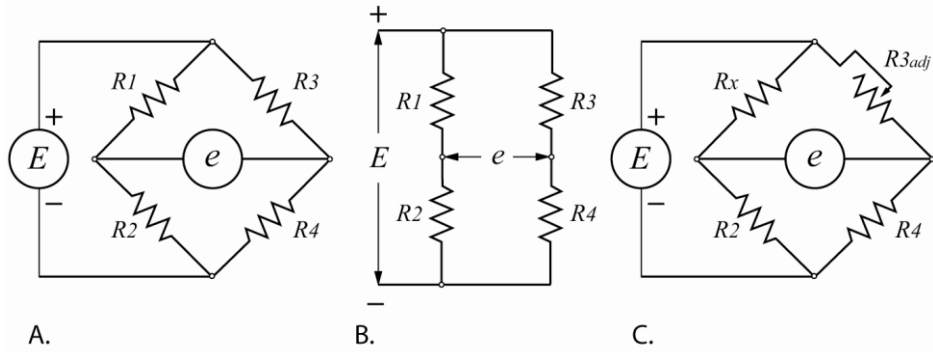


Figure 16.14. A-C. Wheatstone bridge. A. and B. are equivalent circuits. E (italic) is the excitation voltage (produced using an external source) and e (italic) the output voltage (measured with a voltmeter). The principle of the bridge is that e (italic) reacts sensibly to any imbalance in the resistor system. C. is the circuit when the value of an unknown resistance (R_x) (italic) is determined with an ancillary adjustable resistor (R_{3adj}) $\rightarrow$ (R_{3adj}).

The output voltage (add) $\rightarrow$ (e) is obviously nil when $R_1 = R_2 = R_3 = R_4$. It is, however, also zero when $R_1 \times R_3 = R_2 \times R_4$. Such a state is called “balanced,” that is, no electric potential develops on the voltmeter terminals. When one of the resistors changes in value, the voltage will be altered accordingly. At this time, two approaches are possible. The “voltage” method consists of extracting the gauge’s resistance (add) $\rightarrow$ (taken as R_1) from the following relation.

$$e = E \frac{R_3}{(R_3 + R_4)} - \frac{R_2}{(R_1 + R_2)}$$

E is the excitation voltage and e the output voltage.

Expressed somewhat differently:

$$e = \frac{1}{4} E \left[\frac{\Delta R_1}{R_1} + \frac{\Delta R_2}{R_2} + \frac{\Delta R_3}{R_3} + \frac{\Delta R_4}{R_4} \right], \text{ which for one resistor reduces to}$$

$$e = \frac{1}{4} E \frac{\Delta R_1}{R_1} \text{ hence } \Delta R_1 = \frac{e}{E} 4 R_1$$

Since e , E and R_1 are known, ΔR_1 can be calculated.

The “resistor” method consists of balancing the circuit by using a precision adjustable resistor (Figure–14C) and applying the relation

$$R_x = \frac{R_{3adj} R_2}{R_4} \quad (\text{eq.10})$$

with R_x as the unknown resistor (i.e., the strain gauge) and R_{3adj} as the adjustable resistor. The value of R_{3adj} is then read and substituted into the equation. Needless to say, we would use a “manageable” ohmic range for R_{3adj} , for instance 1000Ω.

Numerical Example

Let us assume a balanced bridge with $R_1(\text{the gage}) = R_2 = 120\Omega$ and $R_{3adj} = R_4 = 1000\Omega$. Upon straining, balance is re-established at $R_{3adj} = 991.73\Omega$. Substituting into eq.8, we may now calculate that the gauge’s resistance decreased to 119 Ω. Using eq.8 and eq.9, we then calculate the strain.

Adjusting R_{3adj} is but the simplest method to balance the bridge. In practice, other, somewhat more sophisticated techniques, are used.

Bridge Structures

Figure 16–14 presents a Wheatstone arrangement in its most elementary form (also called a *quarter bridge*). Yet gauges may also be positioned in a number of other positions on the bridge. We will briefly present two- and four-gauge systems (referred to as *half-* and *full-* bridge circuits).

A first two-gauge system is shown in Figure 16–15. This arrangement has the advantage of increasing the voltage sensitivity to strain (e) by a factor two. The relation then is

$$e = \frac{1}{4} E F (\varepsilon_1 - \varepsilon_2) F: \text{ gauge factor (not force as in Figure 16–15)}$$

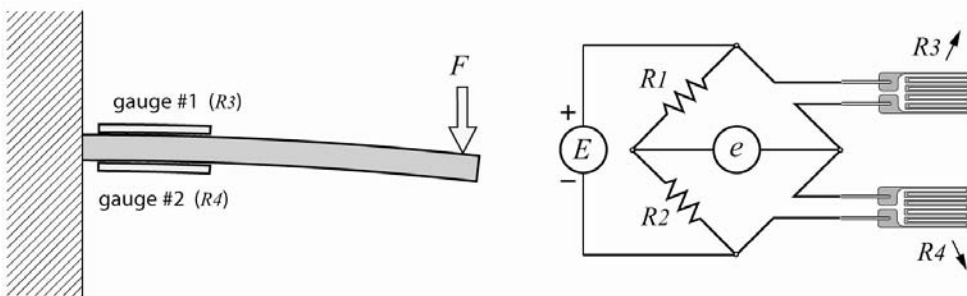


Figure 16.15. Wheatstone half bridge. This arrangement doubles the sensitivity of the gauges. Straining increases the resistance of gauge #1 and decreases that of gauge #2.

A second arrangement is presented in Figure 16–16. This configuration allows for temperature compensation in that it will largely offset the drift in the gauge factor due to temperature shifts. In this instance

$$R_1 = R_2$$

$$R_{3strained} = R_o + \Delta R_{temp} + \Delta R_{strain}$$

$$R_{4unstrained} = R_o + \Delta R_{temp}$$

Using this arrangement, ΔR_{temp} (i.e., the variation in R due to temperature) cancels out as it appears both in the upper and lower member of the resistor ratio that determines the $U_{strained}$ and $U_{unstrained}$ voltages. $U_{unstrained}$ thus functions as a dummy gauge whose sole purpose is to provide compensation for shifts in resistance due to temperature variations. Such arrangements should be used whenever intraoral measurements are performed.

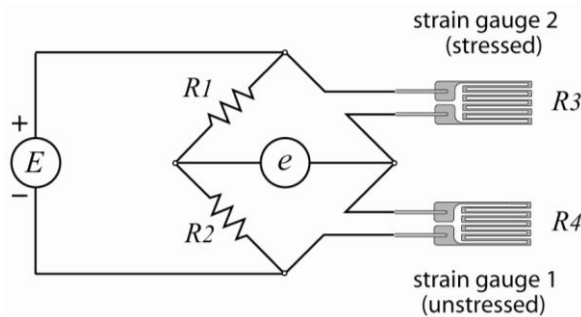


Figure 16.16. Wheatstone half bridge. This arrangement permits temperature compensation.

A full-bridge arrangement is shown in Figure 16–17. This arrangement has four times the sensitivity of a quarter bridge and self-compensates for drifts due temperature if all gauges are in the same environment. It may be intricate to install though, as the gauges must be installed and connected in reciprocal pairs.

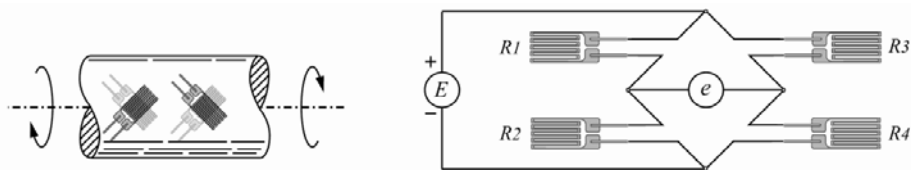


Figure 16.17. Wheatstone full bridge. This arrangement may be used on cylinders subjected to twisting.

Technical Notes

Typical input voltages for Wheatstone bridges are between 5 and 12V, while output readings are in the millivolt range. The input voltage should be kept low to avoid self-heating of the strain gauges due to excessive passage of current. Optimization though is called for as low voltage inputs reduce the sensitivity of the overall system. Due to the low voltage levels, these are prone to electromagnetic interferences. Therefore, amplifiers tend to also amplify the superimposed noise. A solution that is frequently adopted is to use "carrier frequency" amplifiers that convert the voltage variation into a frequency variation (as in voltage-controlled oscillators).

16.2.6. Applications in Implant Dentistry

Strain gauges are indispensable when actual measurements are required. As such, they represent the method of choice when validating finite element models (i.e., computational

abstractions) with experimental data. Still, they have been used to generate "standalone" strain data in the following areas:

- On abutments connected to implants in different configurations and supporting cantilever extensions of various lengths [7].
- On abutments fitted with different attachments to retain overdentures [8].
- On resin models (i.e., bone analogs) surrounding different abutment angulations. [9]
- On resin models fitted with implants (i) to which various attachment-retained overdentures are placed [10], (ii) to which restorations made of various materials are attached [11], (iii) of various connector configurations [12 13]
- On resin models (i.e., bone analogs) fitted with two implants and a three-unit FDP to assess passivity of fit. [14]
- On implant cylinders embedded into resin models to assess (i) the effect of dislodging an overdenture retained with different attachments, [15,16] (ii) the effect of different connectors, [17] (iii) the effect of in-line vs. off-line positioning of the implants. [18]
- On a full-arch cantilevered framework embedded into resin models to assess the strain distribution upon loading. [19]
- On ex-vivo mandibles of minipigs to assess the performance of a new experimental implant design. [20]
- On ex-vivo tibiae of guinea pigs to assess the effect of low frequency loading on bone remodeling. [21]
- On the mandibles of fresh human cadavers fitted with implants to assess the effect of various attachments. [22]
- On the maxillae of fresh human cadavers to assess the effect of grafting on force transmission. [23]
- On an intraoral bar supporting an overdenture to assess the effect of clinical wear. [24]
- On an intraoral framework to assess the effect mixed support (i.e., tooth and implant) and non-rigid connectors. [25]

Strain gauge procedures were also used in the assessment of preload values during screw fastening [26 27].

16.3. Photoelasticimetry

16.3.1. Introduction

Photoelasticity is an optical method to visualize stress fields within a body. The method consists of first duplicating the component under investigation in transparent plastic and then transilluminating the plastic with a beam of polarized light. At this time, the component will start glowing and evenly diffuse the incident light beam.

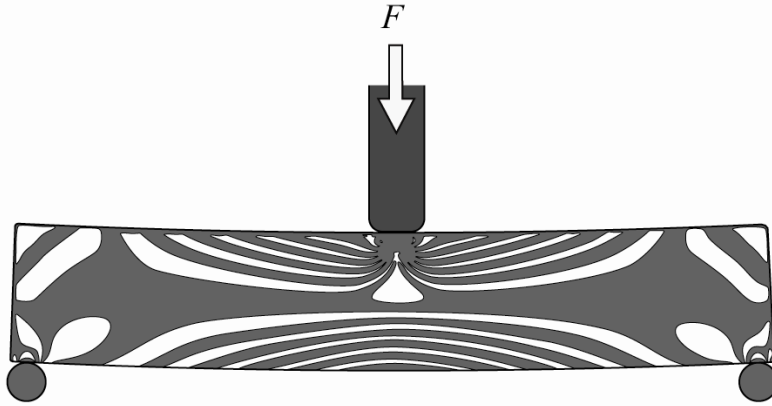


Figure 16.18. Fringe lines. Under photoelasticimetry, the bar (made of photoelastic plastic) is supported on both ends and subjected to load. The fringe lines indicate the distribution of the stresses inside the structure. Adapted from El-Ebrashi 28.

The technique then consists of applying load to the component, thereby introducing slight deformations. At the ångström level, these strains translate into changes in interatomic spacing that alter the light's reflection towards the outside. As a consequence, the component displays color patterns of various shapes – the so-called *fringe lines*. The color pattern is related to the internal strains and thus to the applied force. A black and white representation of the fringe lines (i.e., denoting the stress pattern) generated inside a loaded beam is shown in Figure 16–18. A more detailed account of photoelasticity is provided below.

16.3.2. Principles

Polarized Light

A standard light beam is made of electromagnetic waves that oscillate at any angle relative to the path of light propagation. Such beams are often depicted as a haphazard arrangement that covers the 360 degrees circumference of a cycle. Polarizing such a beam means that only the component of the light beam that oscillates along one single plane is allowed to pursue its course. Polarization is diagrammatically explained in Figure 16–19.

The polarizing effect is obtained by using a special type of filter that is made of grids of parallel lines. The spacing between the lines is set in accordance with the wavelength of light, that is, in the 500-600nm range.

The grid may either be made out of submicron metal wires or of crystals that are stretched on a plastic foil. The latter is the principle of the Polaroid film. Some natural crystals also present a polarizing effect on incident light beams. Such crystals are referred to as being *dichroic*.

Note that the brightness of a polarized light beam can be modulated by interposing a second filter. If the second filter's polarization axis is parallel to the first, all the light will pass through. If it is set to 90 degrees, the beam will be arrested entirely. Setting the second filter at intermediate angles thus permits the experimenter to adjust the light's intensity.

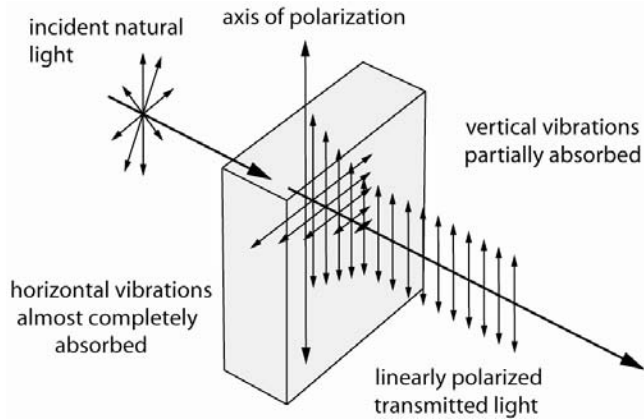


Figure 16.19. Polarization of a light beam. The axis of polarization (AKA “privileged axis”) of the polarizer determines which of the incident light components pass through.

Birefringence

Refraction (i.e., “refrindex”) is the change of direction when a light beam enters a translucent medium. The change is due to the difference in the travelling velocity of the light waves as they pass from the atmosphere into the solid according to the relation

$$\eta_1 \sin \alpha = \eta_2 \sin \beta$$

where η is the index of refraction of the respective media (i.e., the speed of light through vacuum divided by the speed of light through the material) and α the angle of incidence and β the angle of refraction.

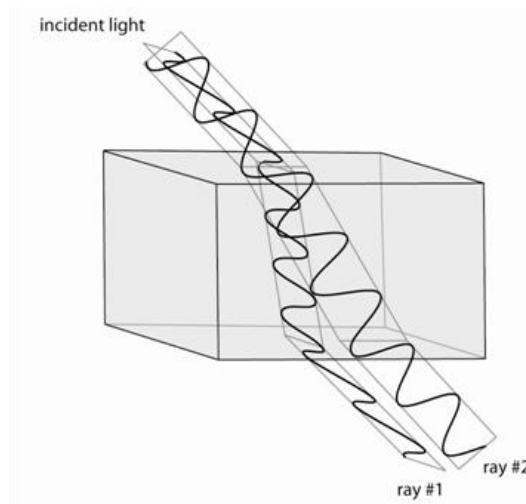


Figure 16.20. Birefringence (AKA “double refraction”). The incoming light beam enters an anisotropic material such as calcite or quartz. Note how the two orthogonal components of the beam are refracted according to two different indices of refraction.

Birefringence (or *double refraction*) is said to occur when the translucent material develops not one but two indices of refraction (η_1 and η_2) and therefore splits the incident light into two beams, each travelling at different velocities (Figure 16–20).

To simplify matters, in the following discussion, we will assume *plane stress*, that is, stress conditions in two dimensions only. This condition prevails when plate-shaped components are tested, that is, when the thickness of the component is substantially less than its overall dimensions.

Stress-Optic Law

In photoelasticity, one uses a transparent (or nearly so) material that becomes birefringent when stressed and whose birefringence augments when the stress is increased. The determining aspect is that the two light beams thus generated are each parallel to a direction of principal stress (σ_1 and σ_2), that is (by definition), orthogonal to each other. The difference in magnitude of both principal stresses is related to the difference in indices of refraction by the relation:

$$(\eta_1 - \eta_2) = C_B (\sigma_1 - \sigma_2) \text{ (the “stress-optic” or Brewster's law)} \quad (\text{eq.11})$$

C_B is called the “stress-optic constant” and its units are “Brewsters.”

It follows that the difference between principal stresses is proportional to the difference between both indices of refraction, that is $\Delta\eta$.

Retardation

As stated, light travels at different velocities along index η_1 and along index η_2 and thus causes the slower beam to lag behind the other. This delay causes a shift in the light's phases and is called *retardation*. Retardation is in the nanometer (10^{-9}) range and denoted as δ . δ is related to $\Delta\eta$ via the relation

$$\Delta\eta = \frac{\delta}{th} \rightarrow \Delta\eta = \frac{\delta}{th} \quad (\text{eq.12})$$

where th is the thickness of the component.

Owing to eq.11, the stress may be calculated as:

$$\sigma = \frac{\delta}{th} C_B \rightarrow \sigma = \frac{\delta}{th} C_B \quad (\text{Eq.13})$$

with:

$\sigma = \sigma_1 - \sigma_2 = \text{stress [Pa]}$. Order of magnitude 10^6 (i.e., MPa).

$\delta = \text{retardation [m]}$. Order of magnitude 10^{-9} .

$th = \text{component thickness [m]}$. Order of magnitude 10^{-3} .

$C_B = \text{stress-optical constant [Pa}^{-1}\text{]}$. Order of magnitude: 10^{-12} .

16.3.3. Polarimetry

After the light beam has been polarized, sent through the strained specimen and refracted, it must be "reassembled" so that retardation can be assessed visually. This is achieved by interposing a second polarized grid called the "analyzer." The experimenter then rotates the analyzer relative to the original plane of polarized light, thereby modulating the light that passes through. By doing so, the 1 and 2 components of the beam interfere, and the specimen glows in various patterns of color – the *fringes*. Fringes are distributed as dark bands separated by various color spectra thereby forming first, second, third, etc., order fringes.

The method can also be utilized for quantitative assessments by determining δ (eq.12) and integrating the value thus found into eq.13. However, interpreting a colored fringe pattern resulting from polychromatic light is nearly impossible as the overlap of wavelengths in different orders prevents their accurate interpretation. Therefore, a monochromatic light beam of 570nm wavelength – the so-called *tint-of-passage* – is used. At this wavelength, the color effect vanishes, and sharply delineated dark/light narrow fringes are obtained.

In practice, the monochromatic beam is first polarized and then sent through a special crystal called a *quarter wave plate* or *circular polarizer*. This device works by causing a quarter wavelength (90 degrees) phase shift between the two orthogonal polarization components of the incident light wave (Figure 16–21).

The object of this 90-degree shift has to do with two optical phenomena termed "isochromatics" and "isoclinics." *Isochromatic* fringes signal zones in which the internal stress state is the same, more specifically zones in which the difference between σ_1 and σ_2 is constant. *Isoclinic* fringes appear when the specimen is oriented so that the axis of polarization of the light beam coincides with either of the principal stresses. This property provides the experimenter with an indication regarding the direction of the principal stresses. The physics of the system is such that isoclinic fringes can be removed from the image when quarter wave plates are interposed, and the analyzer is rotated into proper position. This grandly facilitates the interpretation of the fringe pattern. Figure 16–22 diagrams a full polarimetry setup.

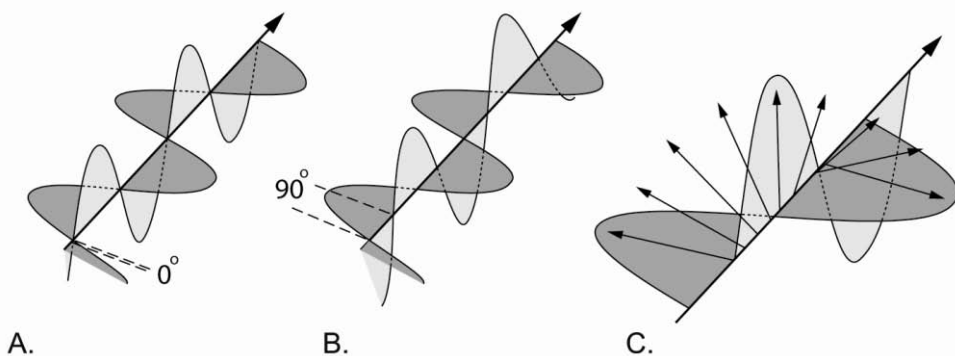


Figure 16.21. A-C. Quarter wave plate. Relative to natural light –phase shift = 0 degrees (A.), this device creates a 90 degrees shift between the light's orthogonal sinus waves (B.). This imbalance causes the light to behave as if it were rotating around an axis instead of vibrating in a single plane (C.). This rotational progression helps the beam in "finding its way" towards the axes of the object's principal stresses.

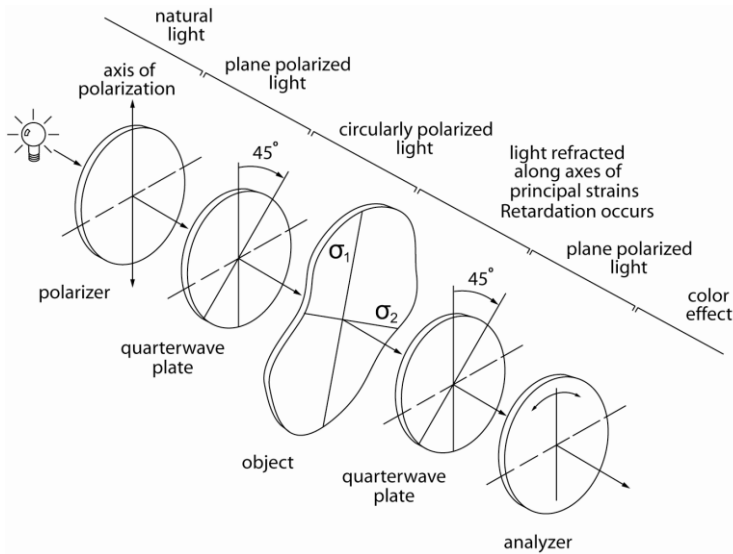


Figure 16.22. Polarimetry setup. The four indispensable elements for polarimetry are a light source, the two polarizers and a birefringent object. By interposing a quarterwave plate, the polarized light is given a corkscrew-like motion which optimally penetrates the object. Upon straining of the object, retardation will occur and colored lines will be visible. The light's phase shift is reverted to plane polarization when the phase is again shifted by 90 degrees in the second quarterwave plate. The fringe effect is obtained by rotating the polarization axis of the analyzer.

As a first qualitative evaluation, zones with tightly packed fringes indicate stress concentrations. Conversely, widely spaced fringes separated by uniform colors denote small stress gradients. Further (i) in line with eq.13, the increase in stress is linearly proportional to retardation and (ii) every dark zone, that is each new fringe order, denotes a phase shift amounting to an integer multiple of the wavelength (also noted as n times 360 degrees). Stated differently beam 2 lags one, two, three or more full phases "behind" beam 1 and therefore the nominal stress is increased by one, two, three or more times. Still, the quantitative analysis of photoelastic fringe patterns requires special competencies to the extent that a number of ancillary aids, techniques and software were devised to assist in the assessment of these data [29-31].

Alternate Methods

The above basically describes testing under plane stress conditions, that is, photoelastic principles as applied to plate-like components. However, the majority of biomechanical issues arises within volumes that are tridimensional and in which the "thickness component" greatly affects the resulting stress patterns. In these instances, although cumbersome, three-dimensional analyses are possible—first, as a qualitative assessment of the stress distribution under load and, second, by using two refinements of the technique, that is *stress freezing* and *surface coatings* [32].

Stress-Freezing

Consists of first placing a transparent three-dimensional body under load, then "locking" the stress pattern into the plastic and, finally, cutting the body into thin slices for interpretation. Each slice is then analyzed as a planar specimen.

More specifically, first a three-dimensional model of the components and/or the surrounding tissues is fabricated out of Plexiglas, polycarbonate, epoxy resin or any other transparent material that exhibits birefringence. Then the model is loaded to the desired level thereby producing fringe patterns. While maintaining the load, the model is heated to a hold-time temperature that is specific to the material and the conditions of loading—a treatment that in effect "anneals" the material and removes its internal stresses. Finally, the model is slowly cooled back to room temperature. The fringe pattern associated with the elastic stress distribution is now locked into (i.e., "frozen") into the model. Then the externally applied load is removed, and the three-dimensional component is carefully cut into slices. When the slices are placed into the photoelastic setup, the embedded fringe information can be recovered. Last, the data from all slices are reassembled and a full three-dimensional view of the stresses is obtained.

Surface Coatings

The photoelastic coating method extends the photoelastic method to the direct measurements of surface strains on structural components. [33] To this end, a sheet of transparent material is cemented onto the surface under scrutiny. To permit light reflection and the development of fringes under load, the sheet must be bonded with a reflective adhesive and the surface must be optimally polished (roughness creates incoherence in the fringes). When the component is loaded, the photoelastic coating will be deformed along with the surface of the structural component, and birefringence will develop in the coating. Using a reflection polariscope, the fringe orders can be recorded and then used to determine the difference in the principal strains (or stresses) on the surface.

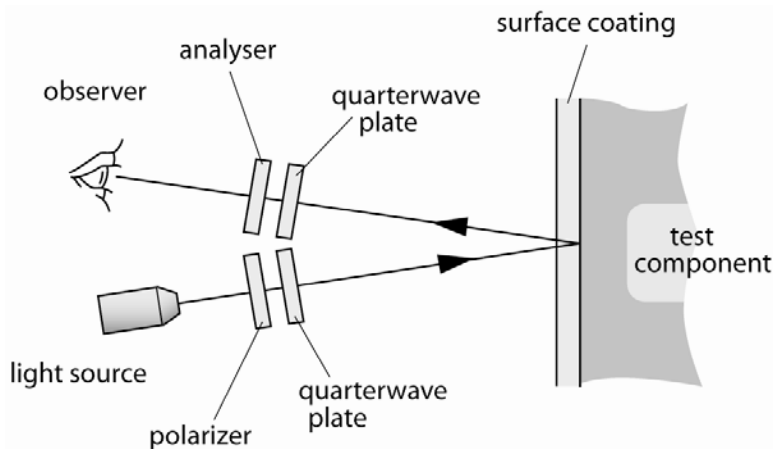


Figure 16.23. Surface coating method. Reflection polariscope. Note the analogy with Figure 16–22 (adapted from Khan and Wang [33]).

16.3.4. Advantages and Drawbacks of the Method

Photoelasticity is a fairly intuitive method for assessing stress distributions inside a component. While it can be used as a fully quantitative method, it essentially aids in determining the stress concentration around sharp internal angles. Relative to numerical

procedures using finite elements, photoelasticity is suitable for assessing stress distributions in the vicinity of abrupt changes in geometry, that is, zones in which the meshing of the finite element model is at pains in duplicating the intricacies of the volume under investigation. In these applications, photoelasticity can be used to determine the stress concentration factor at a given location of the component. When used quantitatively, the method provides reliable full-field values of the difference between the principal stresses in the plane of the model.

On the downside, photoelasticity requires rather tedious calculations to separate the values of principal stresses at a general interior point. Also, photoelastic models lead to oversimplified models when addressing most biomechanical issues, as they cannot properly address differences in material properties of the structures interacting in the mechanical continuum. [34] Last, the method is tedious and time-consuming for three-dimensional work. [35] While photoelasticity still has its proponents, the method is now largely superseded by numerical analyses using finite element models.

16.3.5 Applications in Implant Dentistry

As a first principle, one should remember the following:

- An increase in the fringe order indicates higher stress.
- Closer lines indicate more concentrated stress (Figure 16–24).

In most dental applications, the experimentalist will attempt to locate and assess the intensity of stress concentrations. When affected, such zones are potential sources of failure due to stresses that exceed the yield strength of the material. This information is derived from the isochromatic fringe patterns. In these instances, the isoclinics are of no use and should be eliminated using quarter-wave plates. [37].

It is Zak (1935) who is credited with the first studies using photoelasticity in dentistry. [38] Zak used sculpted resin teeth with photoelastic properties to assess the areas of pressure and tension in the roots when the crowns were subjected to different types of orthodontic force application. About twenty years later, the method was re-introduced by Mahler and Peyton, who described its application to investigate stress fields on occlusally loaded intact and filled teeth as well as on partial denture clasps [39].

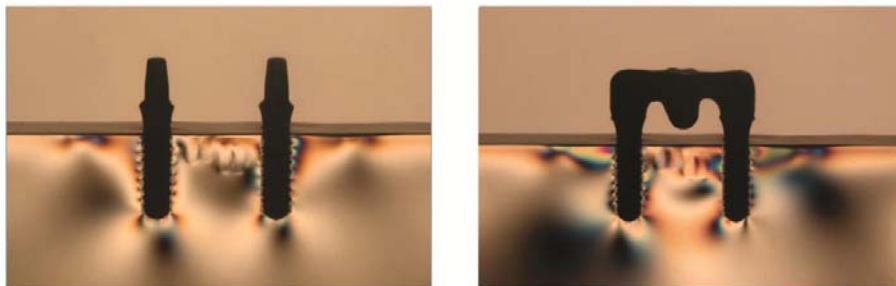


Figure 16.24. Photoelastic response. Two implants are embedded into photoelastic resin. Note how the stress pattern is altered when a fixed partial denture is placed onto the abutments. Pictures courtesy of Dr. M. Karl, with permission of the *European Journal of Prosthodontics and Restorative Dentistry* [36].

In later years, a highly illustrative study demonstrated the stress concentrations in the dentin when threaded dowels were screw fastened into endodontically treated roots [40].

The technique lends itself to the assessment of stress patterns in the bone surrounding endosseous implants. Therefore, a number of studies have dealt with the various parameters affecting the implants' displacements under load. More specifically, the effects of the following were investigated:

- Splinting vs. not splinting restorations on adjacent implants. [41]
- Resin- vs. ceramic crowns. [41]
 - Load transfer between implants and natural teeth when rigidly splinted or joined with semi-rigid connectors. [42]
 - Cementing- vs. screw-fastening the restoration. [43]
 - Connector design (external hexagon, internal hexagon, biconal). [43 44]
 - A 5:4 reduction in platform switching. [45]
 - Implant diameter. [45 46]
 - Implant inclination in the bone bed. [47-49]
 - Adhesion vs. mere apposition of cortical bone around the neck of Straumann ITI, AstraTech and 3I implants. [50]
 - A framework including a marginal gap and combined to parallel and angled implant cylinders. [51]

Combination studies in which both photoelasticimetry and strain gauges were used have been published by Akça and Cehreli [52] and Cehreli et al. [53].

16.4. Finite Element (Numerical) Studies

16.4.1. Introduction

As the name suggests, finite element (FE) analysis is a method in which a complex body is broken down into small portions, the *elements*. This parceling approach is required because the entire body is so intricate that it escapes the classical approach using analytical methods. In the FE method, the elements are first analyzed as separate entities and then progressively re-assembled to provide (i) a global approach of the phenomena involved and (ii) a close-up view of some areas that may particularly impact the function of the whole structure.

To date, the FE method has been applied to the following:

- Thermal analysis, that is, the study of the effect of thermal loads and heat transfer on the design of any mechanical product or components. Thermal analysis applies to nearly any conceivable product ranging from synthetic materials such as metals, polymers or ceramics to fully organic materials and biological samples.
- Electromagnetics, that is, applications in electrostatics, electromagnets and antennas, charged particle beams and high energy magnetic fields for biomedical imaging as well as radio frequencies and microwaves.

- Fluid dynamics. In this context, fluids dynamics is to be construed in its wider sense as it typically comprises convection diffusion, compressible and incompressible laminar and turbulent flow but also the dynamics and resistance of soils.
- Structural analysis, which is the object of this section. In this context, the FE method permits a detailed visualization of the locations in which components bend or twist. The FE method will also indicate the distribution of stresses and displacements.

FE modeling has drastically changed the approach to engineering design in the industry and biotechnological applications. Indeed, this form of "virtual prototyping" provides immediate insight into critical construction parameters thereby decreasing the design cycle and increasing productivity.

Due to its versatility and adaptability, FE has largely superseded photoelasticity in experimental stress analyses. The designer may thus examine a variety of "what if" scenarios in optimizing the design. Conversely, the researcher can examine the effect of material properties and structural interfaces on the stress states inside biological structures. Still, FE techniques belong to a "theoretical world." In this regard, its solutions are only as good as the human mind that has programmed the software.

Regarding terminology, the *FE method* (FEM) refers to the techniques that permit the rapid solving of equations – an aspect that largely translates into to the development of numerical procedures in the form of algorithms. *FE analysis* (FEA) pertains to the application of the FE method to a particular structural problem. As such, it is a goal-oriented process in the mechanical evaluation of components or structures. The difference between FEM and FEA, however, is not always as clear-cut, and both terms are often used interchangeably.

16.4.2. Principles

Anybody under load may be viewed as an assemblage of continuous fields of stresses, strains and ensuing displacements. [54] The complexity of the body's external shape or inner structural heterogeneity, however, is often such that it escapes an *analytical solution* (also called *closed form solution*). "Escaping an analytical solution" means that the stress state inside the body cannot be calculated from elementary engineering formulae. For instance, the equations in Figure 16–1 are an analytical solution to the question regarding the magnitude of the stresses developed in the rod and the beam. Obviously, such formulae exist for geometries that are vastly more complex than the configurations of Figure 16–1; in particular, the effects of acute internal angles and notches as they act as stress concentrators have been expressed in mathematical terms. Still, there is a limit to this approach at which time finite elements are called for.

The principle of FE studies, therefore, consists of subdividing the volume occupied by the structure(s) under investigation into a multitude of small units of elementary shapes, that is, lines, planes or three-dimensional volumes. Each element thus has two to eight (or more) corners that, in the present context, are called *nodes*. Such nodes are the control points of the element as they also carry the information regarding stress, strain and displacement (note that "strain" is a ratio, whereas "displacement" is an absolute magnitude of length).

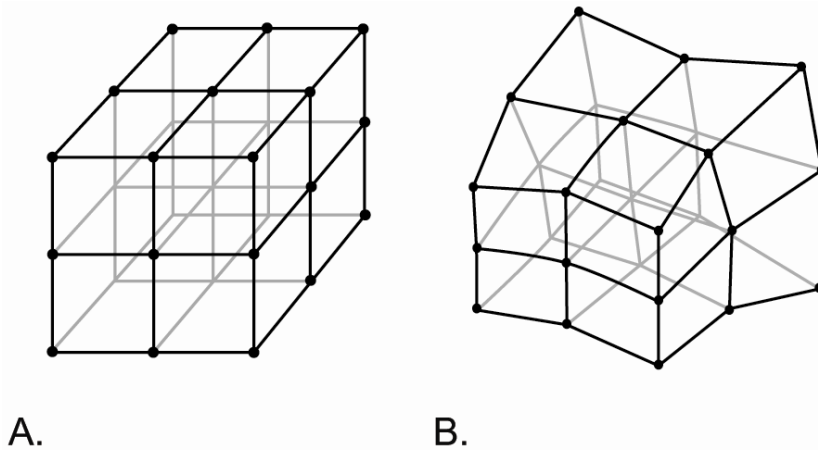


Figure 16.25. A,B. Finite elements under load A. The elements are "at rest," that is unstrained. B. When load is applied to the overall mesh, the local elements start deforming.

Each element is part of a mechanical continuum that is connected to its neighbors via the nodes they share in common. The analytical solutions for such elementary elements are well established and therefore amenable to calculation. The assemblage of all interconnected nodes is called a *mesh*.

Having built a mesh, the experimentalist will now apply a virtual load to one point at the mesh's surface. In reaction, the nodes will start "moving" in response to force application. The magnitude and form of this displacement, though, is by no means equal for all materials and must be set beforehand. The set of equation(s) that determine the displacement form the *constitutive law* for that particular element (or groups of elements). By taking the appropriate derivatives, the calculated displacements thus determine the strains and the corresponding stresses. Note that the load applied can be static (univectorial), a torque, or also include a time factor, that is, some form of vibration.

Integration over the element will provide the *nodal force*. The nodal force contributions from all the elements sharing a common node are then added together. These nodal forces must be in equilibrium with any external load applied to the body. [54]

- The above basically describes the classical approach to FE studies. The systems equations for solid and structural mechanics are obtained using the principles of virtual displacement and work (i.e., the energy equal to force times displacement). [55]
- In the method of weighted residuals (Galerkin method), weighted residuals are used as one method of finite element formulation starting from the governing differential equations.
- Last, the potential energy and equilibrium method (Raleigh-Ritz) involves the construction of assumed displacement fields and uses the total potential energy for an elastic body. Assuming we have determined a function describing the potential energy of a system, then the minimum of that function is the solution of the system.

In the present discussion, we will limit ourselves to the classical method.

The basic principle that underpins the classical FE method is that of virtual work. This principle establishes the relationship between a set of external loads and the corresponding internal forces that develop in reaction to the loads. Indeed, it stands to reason that a loaded beam does not deform indefinitely; so at one point, both force systems must balance out. In technical terms, the external and the internal force systems must satisfy the *condition of equilibrium*. This condition can be expressed using an equation of equilibrium of loads, where the work due to the external loads is equal to the internal virtual work absorbed by the element, that is:

$$\Sigma f \delta = \int_V \sigma \varepsilon d \rightarrow \int_V \sigma \varepsilon d$$

where f denotes the external loads, δ the displacements and $\int_V \sigma \varepsilon d$ the internal force system with its stresses, strains and internal deformations.

The second condition is that of *compatibility*, which relates to the displacements of the nodes and the deformations of the corresponding adjacent elements of a structural system. Compatibility requires that all elements connected to a joint or node point must have the same absolute displacement.

Virtual Springs

Taking the example of a spring, in their most basic form, the force systems may be explained as follows. [56] Let us assume a spring that is suspended on one end and loaded with a weight on the other. The direct relationship between the force (f) (taken as the mass times gravity) and the displacement at the free end (δ) is:

$$f = k \delta \tag{eq.14}$$

where k is known as the *spring constant* – a parameter that expresses the stiffness of the spring.

Knowing the magnitude of the applied force and the value of the spring constant, from eq.14, the displacement can be calculated:

$$\delta = \frac{1}{k} f$$

The above applies to a single spring. Obviously, when the situation implies more complicated force systems, the equations increase in complexity as well. When a number of simple members are interconnected at a number of nodes, the displacement caused by the load can only be described by simultaneous equations. Still, the overall form of the equation remains the same:

$\{F\} = [K] \{U\}$ in the context of FEs, displacements are denoted as u .

In this instance, K (capital K) expresses the stiffness of the whole structure. For instance, if our weight were suspended by two springs oriented in different directions and with different k values, the description of the force system would expand to:

$$\begin{Bmatrix} f_1 \\ f_2 \end{Bmatrix} = \begin{bmatrix} k_{11} & k_{12} \\ k_{21} & k_{22} \end{bmatrix} \begin{Bmatrix} u_1 \\ u_2 \end{Bmatrix}$$

In this relation, (u being the displacement) $\begin{bmatrix} k_{11} & k_{12} \\ k_{21} & k_{22} \end{bmatrix}$ is called the *stiffness* or *property matrix*. It is the matrix that defines the geometric and material properties of the springs. Property matrices are a fundamental part of the FE method. These matrices define the intrinsic properties of the system being studied. Needless to say that "real-life" situations require matrices of higher levels of complexity but the inherent principle is similar.

The essence of the FE analysis thus consists of solving equations comprising the externally applied force (known), the stiffness matrix (as determined by the experimentalist) and the corresponding displacements (the unknown).

Considering FE structures as an assemblage of springs helps in visualizing the function of the FE method. Indeed, the stiffness of the springs may be governed by mathematical relations that are not linear (as in the simplified assumption of k being constant in an "ideal" spring). For a nonlinear spring, for instance, the stiffness must be broken up into two parts:

$$K = K_o + K_n$$

where K_o denotes the linear part and is constant and K_n (i.e., the nonlinear part) is a function of u and depends on the deformation. When $u = 0$, K_n is assumed as 0.

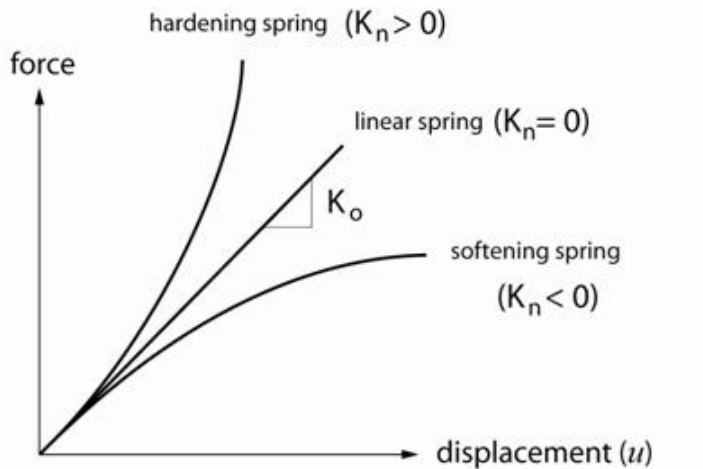


Figure 16.26. Linear and non-linear springs.

With reference to Figure 16–26, in a softening spring, the incremental force F required to produce additional incremental deformation u decreases as spring deformation increases. In contrast, in a hardening spring, the incremental force required to produce a given displacement becomes increasingly greater as the spring is deformed.

For biological systems, the relationship between F and u may become quite complex with zones in which u increases with increasing F and others in which it may stagnate or decrease. Such relationships are described using equations systems known as *constitutive laws*. Obviously, the more complex the constitutive law, the longer the time required to solve the FE force-displacement equations.

16.4.3. Implementing Finite Element Analyses

In the most traditional acception, a model is an (often scaled) copy or representation of an object. Over the past decades, however, the term has evolved to also depict an artificial construct that reproduces the behavior of a complex system for the purpose of investigating and understanding its response. In this meaning, laboratory models may be designed to simulate mechanical phenomena, and animal models would be considered representations of pathological processes. In the same line of thought, the model may also be entirely symbolic, that is, describing a physical phenomenon or a biological process using the symbols of mathematics and computer science. When applied to finite element modeling, this approach results in the three steps shown in Figure 16–27:

- Idealization is the process by which the experimentalist transforms the mechanical or biological system under scrutiny to a mathematical abstraction of the physical reality.
- Discretization is the foundation of FE analyses as it reduces the number of degrees of freedom (we will explain those terms later). The end result of the discretization process is the discrete model (i.e., the mesh).
- Solving consists of iteratively solving differential equations so that a discrete solution is provided.

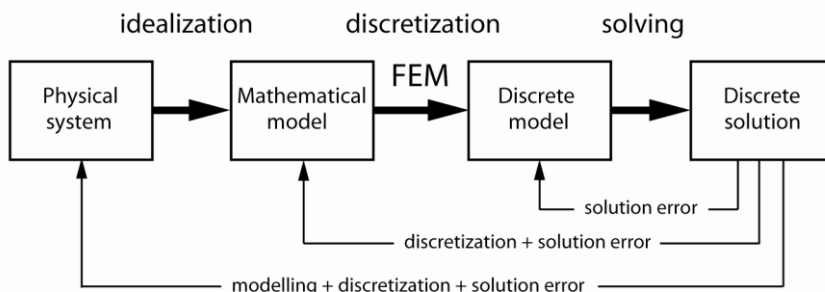


Figure 16.27. Steps of numerical simulation and sources of error Each step in the numerical simulation process induces a source of error. In general, modeling errors are the most important, followed by discretization errors. Solving errors come last.

Implementing an FE analysis largely duplicates the above using a sequence of steps grouped under the following general headings:

- 1) Preprocessing
- 2) Solving (or processing)
- 3) Post-processing
- 4) Validation

To this end, the experimentalist must first choose a software package to use. [57] Choosing from among these involves a multitude of criteria, usually including: analysis versatility, ease of use, efficiency, cost, technical support, training, and the local labor pool that uses a particular software. Table 16–2 lists five companies that are usually considered dominant providers of general-purpose finite element software.

Table 16.2. Leading commercial finite element software

Company	Software	Web site
Autodesk Inc	ALGOR	www.algor.com/
Simulia, Dassault Systèmes	ABAQUS	www.simulia.com
Ansys Inc	ANSYS	www.ansys.com
Siemens PLM software	NX Nastran	www.plm.automation.siemens.com
MSC Software	MSC Nastran	www.mscsoftware.com

Preprocessing

Determine the Outline of the Volume

Preprocessing concerns itself with the generation of a pertinent FE mesh. Such meshes may be either two- or three-dimensional. Two-dimensional meshes extrapolate the third dimension as if it were of infinite length thereby introducing a sizeable discretization error. Therefore, 3D models are preferred. For engineered components such as implant cylinders or their ancillary parts (transgingival rings, abutments, attachments and screws), the technique consists of utilizing a CAD (computer-aided design) software. The components are drawn on the screen according to a set of specifications provided by the manufacturer. Eventually, even complex and intricate volumes may be generated and stored in digital format using sets of 3D coordinates.

CAD programs, however, do not lend themselves to reproducing biological structures whose organizational schemes vastly differ from engineered components. In these instances, the technique consists of generating the pertinent volumes from 3D images. Any technique yielding morphological data in three dimensions is suitable. Surface scanning using laser measurements, computed tomography, magnetic resonance tomography or even synchrotron images may be processed to FE meshes. The resolution of the image is dependent on the imaging device. Larger samples such as human bones would be scanned to a resolution in the millimeter range, while small samples may be imaged to a resolution in micrometers using the latest μ CT (micro-computer tomography) technology. Note that while the resolution in the 2D plane (such as for printing) is determined in pixels (dots per inch), the volume resolution is provided in *voxels* (volumetric picture element).

The intricacy of the techniques lies in the definition of boundaries for a particular biological structure. The human brain uses high-level analytical processes for perceptual groupings and organization in vision. These are based on similarity, proximity, and continuation. Not so for computer generated images in which individual structures must be segregated using a technique called *segmentation*. A number of approaches exist for segmenting images of which the most straightforward is thresholding. Thresholding consists of assigning a threshold value (or an interval) to a grey scale image and filtering so that only those voxels corresponding to the set criteria are depicted. Eventually, one will have isolated one specific structure from the image. Both open source and commercial packages are available. Note, however, that most contemporary procedures are still somewhat unsatisfactory and some manual editing is often required [58].

Generate a Mesh

Having generated a volume, the next step consists of dividing it into numerous volumes of vastly reduced size. In technical terms, the problem domain (often abbreviated as Ω) is viewed as a collection of nonintersecting simple subdomains, the finite elements. Subdividing a domain into elements is called *discretization*.

Discretization is a most sensitive step in the FE process. While most software will readily perform this function, a cognizant approach is indispensable to minimize errors due to wrongful meshing. In a first order approximation, provided certain mathematical requirements are satisfied, the finer the mesh, (i.e., the smaller the element sizes) the closer the approximate solution is to the exact solution. This aspect is intuitively demonstrated in Figure 16–28.

This aspect of meshing, however, has been the object of some debate as the quality of approximation depends on the finite element mesh and on the polynomial degree of the elements. In the classic approach, the polynomial degree of the elements (i.e., their largest exponents) was set to a low value, typically 1 or 2, and the error of approximation was lessened by refining the mesh so that the size of the largest element in the mesh was reduced. In later years, research indicated that keeping the density of the mesh fixed but increasing the number of nodes in each element (i.e., increasing their *polynomial degree*) had important advantages as it better "generalized" the procedure. This difference has entered the literature as the h- versus the p- versions [59] of FE meshes.

At the onset of the 3D FE method, the meshes were made of tetragonal elements. The latter's displacement response, however, was somewhat unsatisfactory and therefore boxes in various shapes were used in later years. Polyhedral meshwork is in research at the moment. [60] An overview of the most used elements is provided in Figure 16–29.

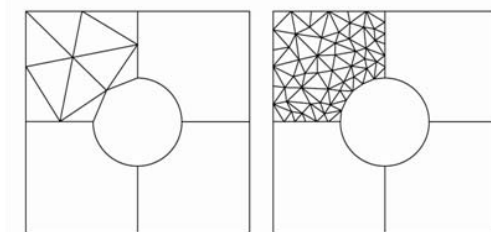


Figure 16.28. Meshing. Note how the finer mesh better approximates the overall contour of the circle.

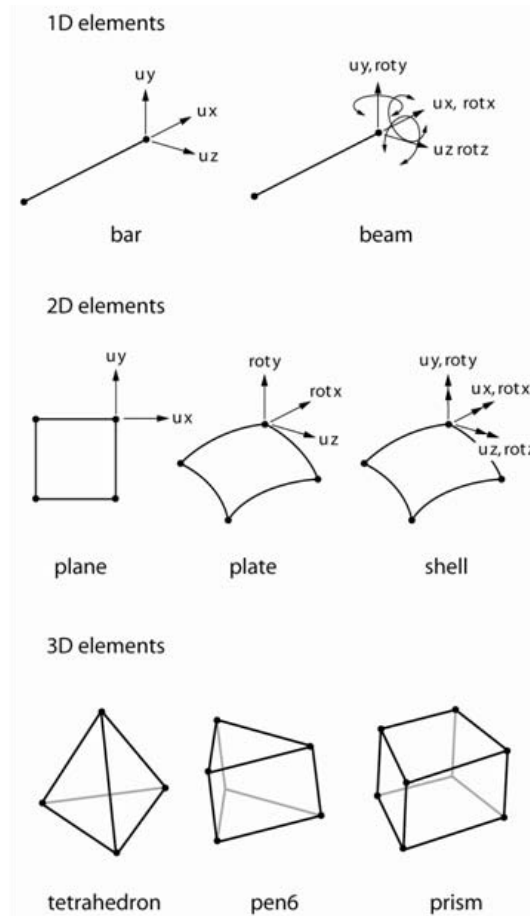


Figure 16.29. Types of elements in FE analysis. One-, two- or three-dimensional elements are available. The nodes may be fitted with translational (u) and rotational degrees of freedom (rot). The more complex the element, the more computing time is required. • : nodes.

Impart Properties to the Mesh

At this time, the mesh must be assigned its properties, that is, the manner in which it will react to an externally applied load. Each element is bordered by a series of nodes. These will typically lay at the intersections with other elements but, with higher polynomial degrees, may also be located in between two endnodes. Each node is imparted a number of *degrees of freedom*. In mechanics, a body may move in the three directions of space (i.e., along the x , y and z axes) but also rotate around these axes thus yielding a total of six degrees of freedom. These are set in the $\{U\}$ matrix with reference to the $\{F\} = [K] \{U\}$ relation described above ($\{U\}$ is the generalized form of $\{\delta\}$). In analytical mechanics, each degree of freedom has a corresponding “conjugate” term, which represents a force component. Linear translations will thus transmit linear forces while rotations will impart moments to the system. The degrees of freedom of a node, therefore, also denote the type of forces a node is able to transmit.

In this regard, by definition, the nodes of a beam element permit all six degrees of freedom (Figure 16–29), hence a beam will transmit univectorial forces as well as moments.

In contrast, other elements only move along the x, y and z axes and therefore cannot transmit forces generated due to rotation (Figure 16–29: bar element).

Boundary conditions are the limitations set in the nodes' degree of freedom. These boundary conditions describe how the model is attached and interacts with its surroundings. A basic example would be that of a body resting on a surface and therefore preventing movements in the direction of the surface. These boundaries are necessary as otherwise the body would be floating in space and, under the action of load, would not suffer any deformation but would merely move around as a rigid body.

Boundary conditions are incorporated into the model in the form of elements that simulate rigidity, mass, springiness, damping effects, relative motion or gaps (i.e., non-adhesion).

In solid mechanics, possible boundary conditions are the following:

- Restrictions in displacements, typically set in terms of degrees of freedom at the boundaries of the FE mesh to define rigid support.
- External forces and moments. These are loads that concentrate on nodes at the exterior of the FE mesh.
- External pressures distributed on the outside of the FE mesh.
- Inertia, that is, loads that affect the entire structure (ex: during acceleration or rotation)
- Temperatures to denote the effect of thermal expansion or contraction.

At this time also, the material is imparted its properties, more specifically the modulus of elasticity and Poisson's ratio. A criterion for failure can also be incorporated. Further, the properties may be distributed homogeneously inside the body, at which time it is termed isotropic, or inhomogeneously when measured along different axes and therefore determined anisotropy. Last, the properties may follow a linear or a nonlinear function (Figure 16–26). Among the nonlinear functions, they may be mathematically simple (for instance a quadratic function) or a more complex relation.

Needless to say, anisotropy and nonlinearity considerably increase the complexity of the model and computing time. Still, they definitively better duplicate biological conditions. [61] A short comparison between linear and nonlinear relations follows [62]:

- Load-displacement

With linear properties, the relation varies as a $y = \alpha x$ function. The stiffness matrix is a constant. By definition, the procedure is conducted within the framework of linear elasticity in which the displacements are small and the corresponding changes in geometry can be neglected. When taken in terms of stress, the relation with strain follows Young's modulus (taken as a constant) up to the proportional limit.

When the properties are nonlinear, the stiffness matrix varies as a function of displacement. Strain is related to stress via a complex function (not a mere constant).

- Reversibility

The structure reverts to its original state upon removal of the externally applied force. Although this is not strictly linked to nonlinearity, most nonlinear materials incorporate some form of hysteresis, that is, their response is time dependent as the materials store and release energy.

- Computing time

Computing time is small for linear relations but may be exceedingly large as the complexity of the relation and the number of elements increase.

The *finite element model* is a mesh fitted with all pertinent constraints, boundary conditions and constitutive laws.

Processing

At this time, the software takes over for *processing* (or *solving*). As stated above, the finite element mesh is parameterized to establish the force-nodal displacement relation. Via the interpolation scheme for each element, the displacement anywhere in an element can be computed according to the number of each node's degrees of freedom. Introducing displacement into the displacement-strain relation, we may express strain in terms of the nodal displacement, the element interpolation scheme and the matrix of differential operators. Further, the potential energy of an elastic body is expressed as the integral of strain energy stored upon deformation, which must balance the integrals of work done by external forces (these are dependent upon the displacement field). Using the relation between displacement and strain, the potential energy of the system can be expressed in terms of nodal displacement.

The aim being to solve the thousands of $\{U\} = \frac{\{F\}}{[K]}$ equations corresponding to each of the elements. To this end, the FE solver proceeds through a series of steps that may be summarized as follows:

- 1) Generate element shape functions.
- 2) Calculate master element equations.
- 3) Establish transformation matrices.
- 4) Map the equations for the elements into a global system.
- 5) Assemble all element equations.
- 6) Incorporate boundary and initial conditions.

After Step 6, the solver starts with its solution procedures. It solves the assembled system of equations to determine the primary unknowns, that is, the nodal displacements and the secondary unknowns, i.e., the values of stresses and strains. For linear relations, it will do so in one step with no iterations. For nonlinear relations, it will proceed by applying the load in small increments thereby verifying that equilibrium is reached at each iteration. This process may be pictured as if the solver were stepping through a geography of peaks and valleys. In a few instances, the solver may get stuck in a local minimum and therefore is said to "fail to

converge." Therefore, solving nonlinear relations requires constant monitoring on the part of the experimentalist.

Processing FE meshes in excess of 10^5 - 10^6 elements is computer intensive. As such, it requires high-performance workstations or clusters if computing time is to remain within manageable limits.

Postprocessing

This is the last step of the process and consists of *verifying* and displaying the results of the analysis. First, the log file generated by the software should be searched for warnings or errors. Then restrained nodes should be examined for balance of applied and reaction loads. Lack thereof invites suspicion regarding the validity of other zones. Deviations among adjacent elements in terms of strain energy, density and stress also indicate potential flaws. Huang et al., for instance, applied a convergence test to verify their model. Differences of more than 6% in von Mises stresses between two adjacent elements were taken as requiring further meshing [63].

The second step is to conduct a procedure referred to as *sensitivity analysis*. Sensitivity analyses provide answers to the following questions: (i) "Which of my FEM input parameters have the greatest influence on my prediction quantities of interest?" and (ii) "How will small errors in my input parameters affect the accuracy of my prediction?"

Then the results themselves are considered. Stresses, being tensor quantities (Figure 16-3), can hardly be visualized using one single form of output. Therefore, the investigator may choose scaled color plots that indicate compressive/tensile stress levels or von Mises stresses. *Von Mises* stresses are a particular combination of all the principal stresses and are a widely used criterion for failure of ductile materials. The criterion is met when von Mises' stresses exceed the yield strength of the material even though none of the principal stresses taken individually does so. Some software also generate plots of the direction of principal stresses. Last, the model's overall displacement may be displayed by superposing the deformed shape over the original configuration. When available, dynamic viewing and animation greatly aid in understanding the deformation patterns of the structures under scrutiny.

More often than not, computational errors or needs for amendments will be apparent in the output. In these instances, adaptive remeshing is called for. Adaptive remeshing consists of generating a denser mesh in zones of brisk or atypical variations in stress levels and a coarser arrangement in areas where only smooth gradients are observed.

In engineering, a further step in postprocessing is to automatically modify the mesh in zones of structural weakness until a predefined criterion of resistance is met. This of course does not apply to investigative research as in biology.

Validation

At this time, the FE analysis still is a theoretical construct. Although the mesh has been optimized, pertinent material parameters were introduced and the solution converges, a validation procedure will lend its final credibility to the model. Colloquially, the procedure consists of assessing how closely the FE data match the "real-world" response of the structures under investigation. Unfortunately, validation is often overlooked and most reports present their results without indications as to verification, sensitivity analysis or validation.

The validation procedures essentially boil down to subjecting the structures to well-defined load vectors and assessing their deformations. The values thus measured should

closely duplicate the values predicted by the numerical analysis. Evidently, especially for biological systems, the match will never be perfect but the deformation patterns and magnitudes should be coherent.

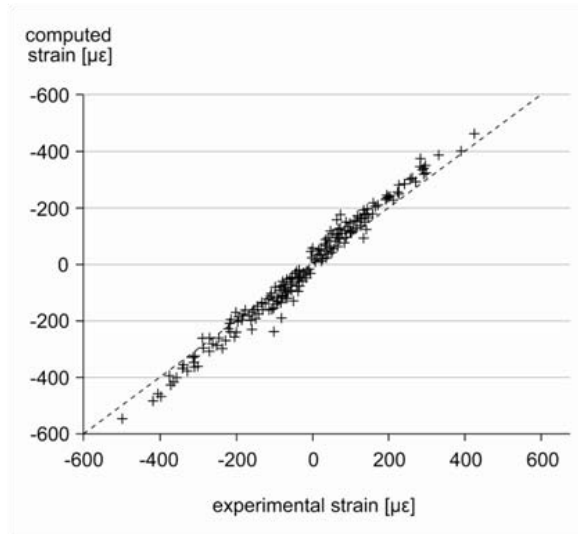


Figure 16.30. Validation Experimental validation of a tibia model. A human cadaveric tibia was loaded and the strains were recorded (i.e., “experimental strain”). These data were then compared to the values computed using FE analysis. Data from Gray et al [65].

One first approach is to determine deformation using imaging techniques, for instance, in assessing the details of tissue deformation in the contact zone between a soft tissue and a surgical scalpel [64].

The second, and most often used approach, is to use strain gauges. If the direction of the principal stress is known, single linear gauges will be used. In case of doubt, rosette gauges (Figure 16–13) must be installed. One such comparison designed to validate the deformation predicted by a tibia model is presented in Figure 16–30. As shown, the measured strains were slightly off-axis but still very much in line with the numerical predictions.

In both instances, only deformations or strains that develop at the surface can be measured. Still, it is assumed that a reasonable match in these areas may be extrapolated to pertinent predictions in non-accessible areas as well.

16.4.4. Advantages and Disadvantages of the Finite Element Method

In spite of it becoming more effective every year, the FE method is still flawed with some pitfalls that should be borne in mind when engaging into FE analyses.

The FE method can properly model [66]:

- Bodies of inhomogeneous materials
- Bodies of nonisotropic materials
- Intricate geometries

- Multiple load vectors
- Complex restraints
- Special material effects (i.e., plasticity, creep, swelling)
- Special geometric effects (large displacements and rotations, contact conditions)

On the downside,

- Although it may allow more general conclusions, an analysis only applies to a specific problem. By design, general closed form solutions are not provided.
- The analysis only provides an approximation to a theoretical closed form solution.
- Experience and judgment are needed to construct a pertinent FE model.
- The technique is susceptible to modeling errors introduced by the user, for instance, a poor choice of element types, distorted elements and inadequate modeling of the geometry.
- The technique is susceptible to material and other nonlinearities.

16.4.5. Applications in Implant Dentistry

Initial publications on finite elements in implant dentistry appeared in the early 1970s. In their 1973 work, Tesk and Widera concluded that an encapsulating membrane of soft tissue greatly reduced the stress concentrations in the bone surrounding an implant. [67] The latter should come as no surprise as the issue of the bone-implant interface was largely unresolved at that time. Three years later, Weinstein et al. devised a two-dimensional FE model of an endosseous 2mm diameter implant. The implant stem was coated with a 1.75mm layer of CoCrMo spherical particles in between which the bone could grow. The model was validated by an in-vivo experiment in the dog [68].

In the wake of these publications, FE analyses gained considerable momentum and led to a host of data in the past years. These are extensively reviewed in several publications to which the reader is referred. [69-71] To provide some examples of current applications, an excerpt of the recent literature follows.

Implant Geometry and Surrounding Bone

Degerliyurt et al. compared stress distribution among the standard (i.e., cylindrical) ITI Straumann-, the stepped cylindrical Frialit-2- and the cylindrical-tapering Calcitek designs. They found that the stepped cylindrical implant dissipated stresses more evenly than the two other designs [72].

Eraslan et al. [73] compared four thread profiles (V shape, asymmetrical buttressed V oriented occlusally, asymmetrical buttressed V oriented apically and square shaped), which were placed on the same (ITI-Straumann-like) implant cylinder. Von Mises stresses were similar for all configurations. V-shaped threads yield the lowest stress intensities upon loading.

Caglar et al. compared three different zirconia implants, that is, the Z-Systems, the Ziterion and the White Sky (Bredent Medical) implants. [74] Although all three systems'

responses were fairly similar, the Z-Systems generated the highest stresses in the surrounding bone bed.

Li et al. attempted to determine the optimal combination of implant length and diameter in type IV bone. Implants of similar geometry (i.e., ITI-Straumann-like threaded cylinders) were modeled. Working under the assumption of stress minimization, the authors concluded that the diameter should exceed 4mm and the length 9mm for an optimal performance in low-density bone [75].

Guan et al. [76] conducted a sensitivity study of their FE model to five variables: implant diameter, implant length, cortical bone thickness, Young's modulus of cortical bone and Young's modulus of cancellous bone. In this study, implant length affected stresses in trabecular bone and the diameter did likewise for the osseous tissue around the implant neck. As an overall conclusion, the authors stated that the applied load had the most effect on the calculated stresses.

Ding et al. modeled ITI-Straumann implants in a situation of immediate loading. To this effect, the contact between the implants' surface and the surrounding bone was taken as "frictional," that is allowing minor relative displacements. The coefficient of friction was set to 0.3 (The coefficient of friction (μ) between two solid surfaces is defined as the ratio of the tangential force required to produce sliding divided by the force applied normal to the interface). Their recommendation was that 10mm implants of at least 4.1 mm diameter should be used for immediate loading applications. In the same vein, Huang et al. generated a series of models in which a variety of geometrical and material parameters were tested with respect to the stresses generated in a grafted sinus. [63] The implants were cylindrical or stepped, either bare or threaded, their length and diameter were varied and they were assigned four levels of interfacial friction ($\mu = 0.3; 0.45; 1$ or bonding contact) to denote various degrees of roughness. The results are in line with expectations in that threads and roughness decreased interfacial sliding but increased interfacial stress. Increasing length and diameter decreased stress but did not reduce interfacial sliding.

Microthreads were the object of Schrottenboer et al.'s study. [77] The microthreads were modeled as V-shaped projections in the sub-millimeter range and located in the coronal 5mm of the implant cylinder. This model indicated that microthreads markedly increased stresses in their vicinity. A radical change in configuration relative to cylindrical implants was investigated by Ihde et al. [78] The implants (referred to as "basal implants") consisted of a cylindrical shaft that was fitted with one or several perpendicular plate-like structures. The authors concerned themselves with the issue of peak stresses when the implants were surrounded by immature bone (i.e., a "soft" contact) and mature bone (i.e., a "hard" bone contact). It was found that the location of the peak stresses at the interface depended on the nature of the contact.

Recognizing the difficulty of multiparameter FE testing, Lin et al. proposed a technique known as "Taguchi method" to reduce the number of possibilities. The method was then applied to the combinations of three implant types x three implant positions x three bone qualities x three loading directions [79].

Rather than the mere testing of existing geometries, in Shi et al.'s study, a structural optimization method referred to as "soft kill option" was used. [80] This technique could theoretically lead to the development of graded materials vis-à-vis their modulus of elasticity; for instance by coating the implants with combinations of titanium and hydroxyapatite. In the

present study, the authors found that a shoulder of increased diameter and of tapered geometry decreased peak stresses.

Implant Positioning

The effect of implant angulation within the bone bed was evaluated by Cruz et al. [81] Fixed prostheses were modeled onto implants, which emerged in a straight line from the mandibular corpus. The test implants were inclined at 35 degrees. Still, no notable differences in stress distributions between both types of implants were evidenced. Similar findings on “tilted” versus “non-tilted” implants were reported by Bellini et al., Bevilacqua et al. [82] and by Zampelis et al. [83] The latter authors also reported that stress levels were much reduced when oblique implants were placed to support a distal cantilever extension. In Las Casas et al.'s study, angled implants generated somewhat less stress than straightly placed implants. [84] Anitua and Orive computed the differences in stresses when implants were positioned midway in a molar edentulous space or when they were positioned under a root (i.e., at either end of the edentulous space). [85] In both instances, the restoration was subjected to a force of 200N mesially and 230N on the distal. In this model, offsetting the implant position actually reduced stresses by about 10%.

The issue of straight line positioning versus staggering of implants was tackled by Abu-Hammad et al. [86] Their model comprised three implants that were either aligned or of which one was offset relative to straight-line alignment. The calculated compressive stresses were essentially similar for all configurations under horizontal and under vertical loads.

Implant Components

Wang et al. [87] evaluated the relationship between the torque applied to a connecting screw and the linear and rotational displacements within the screw-abutment-implant complex. In this instance, the FE model provided indications as to the effect of friction among the different components of the assembly. For instance, torquing the screw head to 32Ncm caused it to rotate by 10.1 degrees clockwise. At the bottom of the screw, however, rotation was 2.4 degrees less. During the process, the screw had elongated by 13.3 μ m. The preload was computed as increasing by 47.9N for every 1 μ m of screw elongation.

Platform switching consists of intentionally reducing the diameter of the abutment component relative to that of the implant cylinder. In the model by Maeda et al., [88] due to the reduced load transfer capacity at the interface, this translated into an increased stress in the abutment or abutment screw.

Akour et al. compared two antirotational designs, that is, the external hex and the tri-channel, cam-type connector. [89] The abutments were screw-fastened onto the implant cylinders. The authors found a notably higher overall stress, contact stress and deflection for the external hex as compared to the cam design.

Implant Function

Naveau and Pierrisnard [90] created a model in which a natural tooth and an implant were connected with a fixed dental prosthesis. In this model, the rigidly set implant acted like a fulcrum and induced the intrusion of the natural tooth. Further, large stresses were generated around the implant neck. Those could be reduced significantly when the FDP's length was reduced and the implant diameter was widened.

In the model developed by Lin et al., two adjacent natural teeth carried restorations. Next to these, a pontic was rigidly connected to a distal implant-borne restoration. This assemblage was then modified to splint both natural teeth and/or to connect the pontic either rigidly or via a matrix-keyway semi-rigid attachment.

The various declinations of the model were then subjected to a variety of load cases (vertical, oblique, localized, distributed). Here also, load magnitude was the main determinant of intraosseous stress. Further, from their model, the authors concluded that a non-rigid connector may more efficiently compensate for the dissimilar mobility between the implant and natural teeth [91].

Rubo et al. investigated variables pertaining to a full arch splint anchored on five anteriorly placed endosseous implants. [92] They altered the length of the cantilever extensions, the E-modulus of the cancellous bone, the abutment height, the length of the implants and the stiffness of the alloy. Intraosseous stresses concentrated around the neck of the implant closest to load application. Increasing the modulus of elasticity of the alloy enhanced stress distribution.

Comparative Studies

In a few instances, authors have compared several methods of stress analysis. Reviews pertaining to strain gauge, photoelasticity and FE analyses were published by Goiato et al. [93] and Assuncao et al [94].

Akça et al. compared the three-dimensional FE models of implants embedded into resin with measurements from strain gauges. In this study, the FE model computed values about half those of the strain gauges. [95] Ozclik and Ersoy [96] addressed the problem of the difference in mobility of implants and natural teeth when connected by a fixed splint. In their model, the connecting FDP was either rigid or included a stress breaker located next to the natural tooth or to the implant.

The three configurations were analyzed by 2D FE analysis and photoelasticimetry. Discrepancies between both methods were evidenced regarding stress distribution along the implant-bone interface. These were attributed to the inability of the photoelastic resin to duplicate the difference in stiffness of the cortical and the cancellous bone. FE models and strain gauge measurements were compared by Eser et al. [97] In this experiment, four implants were inserted into the maxillae of human cadavers. The implants were connected with metal bars and fitted with overdentures. Ninety-degree rosette strain gauges were bonded onto the labial cortical bone in the vicinity of the implants. In parallel, nonlinear models of the implant-bone geometries were constructed. The overdentures were loaded in the first molar regions and the strains were registered.

The correlation between both methods was fair. While in several instances, the data duplicated within $\pm 10\%$, in other load cases discrepancies of a factor two were registered. A three-way assessment was published by Karl et al. [36] on an elementary model comprising an implant-supported three-unit FDP (see Figure 16–24). The authors evaluated the intraosseous strain caused by prosthesis cementation. Strain gauge measurements, photoelasticimetry and FE analyses were conducted. While no actual values were provided, the authors concluded that the combination of all three methods provided the most reliable basis for interpretation.

16.5. Biomechanical Parameters of the Bone-Implant Interface

The biomechanical interactions between bone and implants are complex and only incompletely understood at this time. These interactions may be approached either from their biological end (for review, see Chang et al. [98]) or in terms of mathematical formulations (for review, see Lin et al. [99]). Most FE models clearly function within the framework of implant overload [100] —hence the ubiquitous goal of localizing zones of increased stresses. In only few instances was the hypothesis tested that local bone resorption was actually resulting from lack of bone-to-implant coupling and bone understimulation [101] —a phenomenon known as “stress-shielding” in orthopedics.

Whichever concept ultimately prevails, implant bioengineering requires that the parameters that characterize the interface be determined. Therefore, this section is conceived as a summary of available data.

16.5.1 Modulus of Elasticity and Poisson’s Ratio

Young’s modulus (μ) and Poisson’s ratio (ν) were explained in Figures 16–6 and 16–7. Both enter FE models as prime parameters of constitutive laws. A list of published values for μ and ν is provided in Table 16–3.

Table 16.3. Modulus of elasticity and Poisson's ratio

Tissue/material	Modulus of elasticity [GPa]	Poisson’s ratio	Source
Bone – cortical	14.6 15 12.8-20.1 12.8-17.7	0.33 0.30 0.22-0.42 0.41-0.49	Cowin [106] Rice [102] Ashman [104], Currey [109] Reilly [105], Currey [109]
Bone – trabecular	1.37 1.5	0.3 0.3	Widera [108] Rice [102]
Ti	100 110	0.34 0.28	Akour [89] Sakaguchi [103]
Ti6Al4V	110	0.34	Akour [89]
Gold alloy	100 86	0.28 0.30	Sakaguchi [103] Moffa [107]
Ag-Pd alloy	95	0.33	Powers [111]
CoCr alloy	218	0.33	Powers [111]
Periodontal ligament [#]	0.00007-1.75 [#]	0.3-0.49	Rees [110]

[#] The response of the periodontal ligament is nonlinear and poroelastic. It cannot be expressed by a single value.

Regarding the modulus of elasticity of cancellous bone, the reader should be cautioned that a range spanning two orders of magnitudes (0.08 to 7.93 GPa) has been found in the literature [71] and that authors tend to quote each other in sequence without verifying the original sources [112]. For cortical bone, the range was 5.57 to 22.8 GPa.

Poisson's ratio is a fairly elusive parameter. Note that its theoretical upper limit is 0.5, at which time the material is absolutely incompressible. In case of doubt or when the value is unknown, ν is often arbitrarily set to 0.3-0.35.

The coefficient of friction (dry) of grade 5 titanium is in the 0.3 range. Assuming some lubrication by saliva, a decrease to 0.1 may be assumed [89].

16.5.2. Interfacial Tensile Strength

Data available on the interfacial tensile strength between bone and an implanted biomaterial are listed in Table 16–4. In these instances, plates were implanted into bone and left to heal [113].

Then the plates were lifted off the osseous substrate by a pulling force normal to the bonding interface. Note that the bond strength of bone to the materials is about two orders of magnitude lower than the intrinsic tensile strength of bone.

Table 16.4. Tensile bond strength of bone on implant materials Note the extremely low values. HA: hydroxyapatite; TPS: titanium plasma spray; KG Cera is a "bone-bonding" glass-ceramic. R_a expresses the "mean roughness" of a material

Material	Animal	Interfacial tensile strength [MPa]	Source
Ti ($R_a = 0.48\mu\text{m}$)	Rat	0.01	Skipitz ¹¹⁴
Ti ($R_a = 0.48\mu\text{m}$) Alkali treated	Rat	0.29	Skipitz ¹¹⁴
Ti-6Al-4V	Rabbit	0.18 ± 0.05	Kitsugi ¹¹⁵
Ti-6Al-4V ($R_a = 0.77\mu\text{m}$)	Dog	0.27 ± 0.47	Taylor ¹¹⁶
Ti-6Al-2Nb-Ta	Rabbit	0.17 ± 0.08	Kitsugi ¹¹⁵
Ti-TPS ($R_a = 20\mu\text{m}$)	Monkey	2.7 (1.1 - 4.2)	Steinmann ¹¹⁷
Ti-TPS ($R_a = 175\mu\text{m}$)	Rabbit	3.08 ± 0.68	Pröbster ¹¹⁸
Ti-6Al-4V – Ti_5Si_3	Rabbit	0.18 ± 0.06	Kitsugi ¹¹⁵
Ti-6Al-4V – HA	Dog	2.7 ± 0.82	Taylor ¹¹⁶
Ti-5Al-12.5Fe – HA	Rabbit	1.97 ± 0.29	Gross ¹¹⁹
HA ($R_a = 0.05\mu\text{m}$)	Rabbit	0.85 ± 0.55	Edwards ¹²⁰
HA	Rabbit	1.59 ± 0.72	Hong ¹²²
Ti – HA20Ti	Rabbit	6.5	Chu ¹²³
KG Cera ($R_a = 10\mu\text{m}$)	Rabbit	1.11 ± 0.11	Gross ¹¹⁹
Bone tensile strength		100-150	Cowin ¹²¹

16.5.3. Interfacial Shear Strength

Interfacial shear strength may be tested by pulling, pushing or rotating an osseointegrated cylinder out of its bone housing. Under force application (Figure 16–31), the interface reacts elastically until it mechanically fails. The resistance may thus be expressed as $\tau = f / s$, where f is the shear force and s the contacting surface. Besides s , τ also strongly depends on the surface's roughness.

In theory, push/pull out tests should be applied to implants devoid of macrofeatures such as threads. In orthopedics though, many authors chose to pull-out threaded implants or screws as well. [124,125] An example of a pull-out sample in a rat bone is shown in Figure 16–31. A sampling of available data on threadless cylindrical implants is presented in Table 16–5.

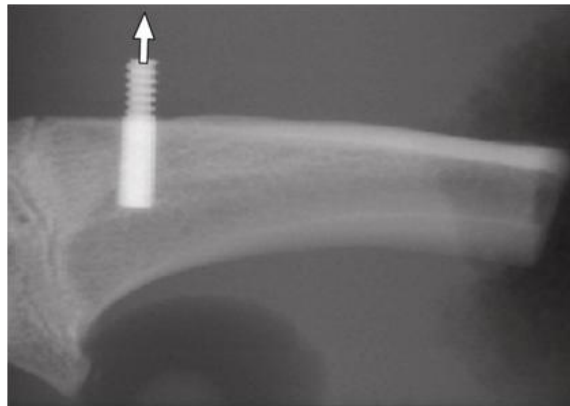


Figure 16.31. Pull out specimen. The specimen features a bioactive portion (i.e., the implant) and a treaded shaft to which it is affixed during the pull-out test. Picture courtesy Dr. P. Ammann.

Table 16.5. Interfacial shear strength. Shear strength was established by driving osseointegrated cylinders out of their bone bed. SLA: Sandblasted, large grit, acid etched. TPS: Titanium plasma spray

Material	Animal	Interfacial shear strength [MPa]	Source
Ti (polished)	Rat	1.14 ± 0.41	Nakgawa ¹²⁹
Ti ($R_a = 1.16\mu\text{m}$)	Dog	1.24 ± 1.05	Nishiguchi ¹³⁰
Ti – SLA	Rat	4.02 ± 0.44	Dayer ¹²⁶
Ti – SLA	Rat	3.57 ± 0.39	Maimoun ¹²⁷
Ti-TPS ($R_a = 101\mu\text{m}$)	Rabbit	6.29 ± 0.80	Pröbster ¹¹⁸
Ti-6Al-4V ($R_a = 0.20\mu\text{m}$)	Dog	0.57 ± 0.38	Nishiguchi ¹³⁰
Ti-6Al-4V – TPS	Dog	2.6 (1.4-3.7)	Jakobsen ¹²⁸

16.5.4. Removal Torque

The removal torque test (AKA *reverse torque test*) is the second variety of shear tests. As the name implies, the implant is subjected to counter-clockwise rotation until breakage of the

osseous bond. In contrast to push/pull-out tests, reverse torque tests are applicable to all near-rotation-symmetric implant configurations, that is, including threaded or tapered geometries. In principle, the removal torque test is the same as the interfacial shear test described above. The essential difference though lies in the external geometry of the implant, which may considerably augment the surface. These studies therefore are comparative in nature and report results in terms of Ncm (i.e., torque) and not MPa (i.e., interfacial shear strength).

Initial reports involving removal torque tests were published in the late 1980s [131,132] and in 1996, Sullivan et al., proposed that a removal torque of at least 20Ncm be set as an indicator of proper osseointegration. As they depend on the configuration of the implant, removal torques may take a full range of values. Typical figures are in the 40 to 60 range, but a staggering 174Ncm has also been reported [133].

A Note on Shear Tests

Shear tests (of the interfacial strength or removal torque variety) are complex in their interpretation as they reflect the added effects of a number of parameters:

- The outer geometry of the implant.
- The surface physics of the implant material (chemical composition, microstructure, free surface energy). [134]
- The implant's surface topography (i.e., roughness), which may range from $S_a = 0.68$ - to $1.75\mu\text{m}$ (S_a is the three-dimensional sibling of R_a) with a concomitant augmentation in developed surface area—the latter increasing from 27- to 143%. [135]
- Bone-to-implant contact, supporting bone mass and bone microarchitecture [136]
- The bone's mineral density. [137]

As such, shear tests should be viewed as an integrative "quality" factor rather than a true material characteristic. Still, when expressed in MPa, σ or τ values may be integrated into finite element models [138].

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Part VI – Clinical Research

Chapter XVII

Introduction to Clinical Research in Implant Dentistry

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17.1. Introduction

Clinical research involving human subjects has as its aim the accumulation of generalizable knowledge for understanding and improving human health and well-being. It is generally preceded by preclinical research to establish some “outcome measures” or “endpoints” of the planned clinical investigation. It involves a set of activities to test a hypothesis, permit conclusions to be drawn, and thereby contribute to the gathering of generalizable knowledge useful to others.

Clinical research should be distinguished from clinical practice, because their purposes and goals are quite different. The purpose of clinical practice is to diagnose, prevent, treat, or care for an illness or condition in an individual, or a group of individuals, with the goal of meeting the needs of and benefiting that individual. On the other hand, in clinical research, investigators recruit patients with the predetermined characteristics, administer the treatment(s), and collect data on the patients' health for a defined period. The subjects of the

research may or may not benefit from participation, but the goal of such research is to serve a common or collective good by generating knowledge useful to improve medical care.

Clinical documentation of dental implants was started in the mid-70s by Professor Brånemark, with excellent long-term follow-up. [1] Recently, several dental implant brands have been well documented for five years or more. [2] However, the dental implant market is undergoing a *rapid* change, and dental implant companies continue to launch new brands without any clinical documentation. Clinical documentation seems unimportant to such companies, because they can market their products successfully without spending time and resources on clinical research. [2] Instead, these companies publish pseudoscientific papers in order to provide some not clinical relevance data only to support their products.

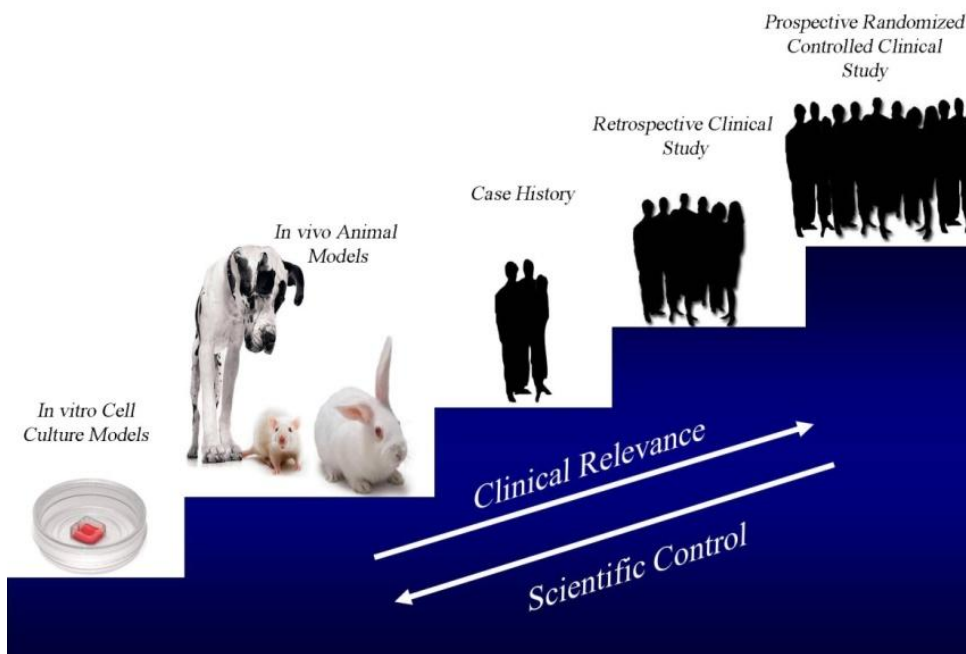


Figure 17.1. Hierarchical approach for screening new materials in dental implant applications.

Further, clinical studies are not needed for most implant or abutment devices according to regulatory agencies of the European Union and United States of America. For example, the US Food and Drug Administration states, “In accordance with the least burdensome provisions of the FDA Modernization Act of 1997, the agency will rely on well-designed bench and/or animal testing rather than requiring clinical studies for new devices unless there is a specific justification for asking for clinical information to support a determination of substantial equivalence.” [3] What is sought is a series of sensitive and controlled preclinical tests that predicts the clinical outcomes and parallels the set of clinical performance parameters. The traditional methods for such preclinical tests are *in vivo* animal models and *in vitro* cell culture models (Figure 17–1). However, from a clinical perspective, the simplest case report is more relevant than most animal and *in vitro* data.

Few randomized controlled clinical trials of oral implants have been conducted to answer specific clinical questions. Among these, the chief questions relate to loading protocols,

implant surfaces and designs, surgical techniques, and implant numbers needed to support different types of prostheses.

Various types of clinical study designs can be employed to investigate the questions about dental implants. Such research can be categorized as non-experimental (i.e., descriptive or observational studies) and experimental (i.e., treatment studies). The former includes case reports and cohort, case-control, cross-sectional, and ecological studies, and the latter includes randomized controlled clinical trials and non-randomized (or quasi-experimental) clinical trials. This chapter provides an overview of these clinical research designs, along with the strengths and limitations of each approach. Further, the general principles of randomized controlled trials are discussed in detail.

17.2. Clinical Research Designs and Protocols

The notion of evidence-based dentistry supposes that clinical decisions will be made after considering the quality of information available in the professional literature. Such information most reliably arises either from standardized reviews of research, such as systematic reviews or meta-analyses, or from directly assessing the original research, and in either case, the conclusions that can be reached are only as good as the research on which they are based. While such research may originate in the laboratory, it is rigorous clinical investigation that will demonstrate the value of various treatment modalities. Whether practitioners are interpreting the literature or planning their own research, knowledge of the study designs available, and of the strengths and limitations of each design, is key to reaching defensible treatment decisions.

Clinical research can be conducted using qualitative or quantitative methods. Qualitative research makes a detailed exploration of a small number of subjects using open-ended questions, often providing information about hard-to-answer questions like “why.” Qualitative methods lend themselves well to exploring concepts, understanding the depth of problems and considering the implications of findings. [4] Quantitative research, on the other hand, focuses on testing hypotheses with large enough numbers of subjects to allow statistical analysis and generalization of results to populations. [5] It can be argued that our understanding of the real-world implications of dental implants could be deepened by better utilizing qualitative research methods, either alone or in combination with quantitative methods. For more detailed information about qualitative research methods, the reader is directed to any of a number of texts published on the subject. [6,7] Nevertheless, because most clinical research related to dental implants has to date focused on quantitative approaches, this section will provide an overview of the various types of quantitative study designs that can be employed to answer clinical questions about dental implants. Such research can be categorized as non-experimental (also called descriptive or observational studies) or experimental (treatment studies). [8] As the name implies, non-experimental (descriptive or observational) studies are intended to provide a description of events where the researcher is observing, not intervening. Experimental or treatment studies attempt to address the issue of causation, with the researcher actively intervening via the treatment provided. The types of quantitative research designs addressed here are summarized in Table 17–1. No one design is suitable for all types of research, and the protocol chosen will depend

to a large extent on the question being asked. For example, non-experimental studies may set the stage for later experiments once more is known about the presence and characteristics of a problem.

Table 17.1. Types of Quantitative Clinical Research

Types of Quantitative Clinical Research	
Non-experimental	Experimental
Case reports	Randomized, controlled clinical trials
Cohort studies	a. Double-blind
Case control studies	b. Single-blind
Cross-sectional studies	c. Non-blind
Ecologic studies	Non-randomized clinical trials (quasi-experimental)

17.2.1. Non-Experimental Clinical Research Methods

17.2.1.1. Case Reports

Considered a starting point for sharing new and emerging issues, case reports focus on an individual patient’s presentation and outcomes. They may be the first step in identifying potential problems that warrant further research and can provide an introduction to unusual clinical presentations. A case report requires enough detail about the patient and the situation in question for others to recognize similarities to patients they have seen. The focus is on discovery instead of proof, [9] meaning that while they are the lowest level of evidence, case reports can be the first step in beginning to establish evidence.

Case reports have as advantages their low costs, convenience, and minimal ethical risks, since treatment is not being either withheld or randomly assigned. They are, however, limited by the potential bias of the author, the lack of generalizability, and the inability to show causation.

17.2.1.2. Cohort Studies

A cohort is a clearly defined group, or population, that shares identified characteristics and is studied over a period of time. [10] This type of study identifies two cohorts, one that has the characteristic being investigated and the other that does not. In this design, it is important that participants are initially free of the condition being studied and vary primarily with respect to their exposure to the identified risk factor or intervention.

Cohort studies can be conducted prospectively, where the groups are selected based on exposure, or lack of exposure, to a risk factor and then followed from the present over time to see which groups develop the condition of interest.

Cohorts can also be studied retrospectively, looking at historical data and identifying cohorts based on their exposure, or lack of exposure, to the intervention or risk factor of interest. As with the prospective design, the retrospective cohort study also compares outcomes from the date of exposure to the present.

Both methods compare the frequency of the identified outcome between the cohort that was exposed to the risk or intervention and the cohort that was not exposed. Prospective studies are typically considered to be preferable to retrospective studies because data collection can be more accurate with respect to exposures, confounders (unknown causes), and endpoints with the former, [11] and data are not specifically collected for the latter, leading to possible missing information or unmeasured confounders. [12] On the other hand, even the retrospective design goes forward in time, and it is substantially less expensive and time-consuming than its prospective counterpart [13].

Compared to randomized controlled trials (RCT), cohort studies are less expensive and easier to undertake, with fewer practical and ethical constraints, and have broader inclusion criteria with fewer exclusion criteria, making their results potentially more generalizable to clinical practice. [11] Cohort studies also allow study of rare risk factors, as well as multiple exposures and outcomes, and they readily show associations between exposures and outcomes [11].

Limitations of cohort studies include the inability to establish causation and, because subjects are not randomized, there can be imbalances in patient characteristics. Likewise, it is possible that confounders may be responsible for the effects noted and not the identified risk or intervention. Selection bias and inability to account for loss to follow-up, leading to possible incorrect inferences about treatment effects, are also disadvantages of cohort studies [12].

17.2.1.3. Case Control Studies

While cohort studies are based on exposure to a risk or intervention, case control studies are based on outcomes. [5] They compare patients who have a condition of interest (cases) with those who don't (controls), looking retrospectively at treatment records for exposure to the risk or intervention to see if there is any relationship between the two. Key to this comparison is ensuring that the cases and the controls are similar in every respect except for their exposure to the condition being studied [12].

Case control studies, also called "case-referent" studies, are advantageous when the outcome of interest is rare and because they can be done relatively inexpensively and quickly. Furthermore, they can be conducted when other study designs are inappropriate, they can assess multiple risk factors, and they are helpful in generating hypotheses that can then be tested using a prospective study design [10].

On the other hand, selecting appropriate controls for a case control study is difficult and may inadvertently introduce bias, undermining the validity of any conclusions reached in the study. Bias is also an issue related to the recall of the condition being studied, where those who have had the condition or treatment may have a more detailed recollection of associated events than those who have not.

To summarize the sometimes confusing difference between a cohort study and a case control study, the former asks if an identified risk or intervention is associated with an outcome, while the latter asks if an identified outcome is associated with a risk or intervention [8].

17.2.1.4. Cross-Sectional Studies

This design involves a survey or overview of a population at a specific point in time, in contrast with a longitudinal design, which studies change over time. [8] The cross-sectional

study is relatively straightforward and quick to conduct, as well as being useful in assessing the prevalence of risk factors and the frequency of defined outcomes for a population. Interview surveys and mass screening programs are two examples of this design.

A disadvantage of the cross-sectional study design is that data about both exposure and outcome are collected at the same time, creating questions about “the temporal relationship of the presumed cause and effect.” [10] This type of study also selects for longer-lasting conditions, which are more likely to be detected by a “snapshot” survey than short-term outcomes that may have come and gone when such a survey is undertaken.

17.2.1.5. Ecologic Studies

Ecologic studies look at a population rather than at individuals, using aggregate data to investigate the relationship between exposure to a known factor and an identified outcome.² Such research may be conducted at a defined point in time (cross-sectional) or over time (longitudinal) and often compares populations in different geographic locations.

The key difference between ecologic and cohort studies is that the former looks only at the population level, while the latter examines individuals.

Ecologic studies are inexpensive and take advantage of existing data, but while they are useful for identifying potential hypotheses, they cannot demonstrate cause and effect because there is no way of knowing whether those who demonstrate the effect are those who were exposed to the risk.

This inability to infer causation is the principle weakness of observational, or non-experimental, research, while the converse, establishing causation, is the main advantage of the experimental study designs to follow.

17.2.2. Experimental Clinical Research Methods

To be considered a true experimental design, a study must include random assignment of subjects to study groups, one of which must a control group, and, ideally, only one variable should be tested. The randomized controlled trial (RCT) is often described as the “gold standard” [14] in clinical research because it meets these criteria.

17.2.2.1. Randomized Controlled Clinical Trials

As described in detail elsewhere in this chapter, the RCT is an experiment where the research subjects are randomly allocated to one of two groups: the control group, which will receive either a placebo or the standard treatment, or the treatment (intervention) group, which will receive the treatment under study. Randomization is the key to the robustness of this research design because it helps to minimize bias, intentional or unintentional, in the assignment of treatment to study subjects, who have an equal chance of being allocated to either group. The notion that the treatment and control groups are the same at the start of the study allows for inferences that the experimental treatment, and not differences between the two groups at the outset, is the cause of any differences observed over the course of the trial.

Another way to reduce bias in an RCT is by preventing participants in the research from knowing the type of treatment provided. One way to assist in masking which treatment is

which is to make the non-experimental treatment appear to be identical to the experimental intervention. [10] Another is to “blind” participants as to the type of treatment given.

Double-blind Design. In this approach, both the research subjects and the investigators are ignorant about which treatment is provided to which group. While this is the best method to reduce bias when treatment is given or received, it is not always possible due to practical or ethical limitations.

Single-blind Design. As an alternative to the preferred double-blind method, single-blinding involves concealing the treatment received from the research subjects, even though the differences in treatment cannot be hidden from the investigators.

Non-blind Design. In some cases, the difference between the standard and experimental treatments cannot be hidden from either researchers or participants.

In the case of both single-blind and non-blind clinical trial designs, it is still possible to reduce bias by blinding those assessing the outcomes and by ensuring that all involved in providing treatment are counseled to avoid inadvertently offering opinions about either treatment to research subjects. It is also important to document which members of the research team generated the random allocation sequence, enrolled participants and assigned them to treatment groups, [15] ensuring that each member of the investigative team is educated as to the importance of adhering to the randomization protocols and treating each intervention equally, both in front of and away from study subjects.

RCTs are most often structured as a parallel arm design with two or more groups, where subjects stay in their assigned groups throughout the trial. In comparison, the crossover design, typically conducted when the results are reversible, randomly assigns subjects to a sequence of treatment. For example, one group will receive the standard treatment followed by the treatment being studied, while the other will receive the two treatments in the opposite sequence. In order to reduce the potential for a carry-over from one treatment to the other, a “washout” period between interventions is typically introduced. This type of trial has the advantage of reducing the number of subjects who need to be recruited, a significant challenge in mounting a clinical trial [16].

If the primary advantages of the RCT are its ability to reduce bias and evidence cause and effect relationships, it is hampered, on the other hand, by its lack of applicability to many research questions and its complex, expensive nature. [17] There are also issues around the external validity of RCT results, related to their translation from the carefully controlled clinical trial environment to the messy real world.

17.2.2.2. Non-Randomized Clinical Trials (Quasi-Experimental)

The quasi-experimental design typically does involve a comparison group, but subjects are not randomized. [5] Sometimes, this is because groups are formed without any control by the researcher, and sometimes it is more practical or convenient to arbitrarily construct the groups. In other respects, this type of study proceeds as usual, comparing a variable between the groups. Quasi-experimental and experimental designs are similar to the extent that they both introduce an intervention rather than simply observing subjects over time [8].

This type of study is most applicable in assessing the social aspects or impacts of an intervention, where pre-selection and randomization of groups is particularly challenging. Of course, the lack of randomization introduces the possibility of significant immeasurable differences between the groups being studied and limits the validity of conclusions that may be drawn.

17.3. General Principles of Randomized Controlled Trials

Clinical trials represent a central part in pre-clinical testing of medicinal products (drugs) and medical devices (e.g., implants). To allow a risk adopted *evaluation* of medical devices, the EU directive classifies medical devices according to their time in use, their invasiveness, and implantability. The higher the classification the greater the level of assessment required. It is the “Intended Purpose for Use,” assigned by the manufacturer to the device that determines the class of the medical device. This means that two instruments that have the same composition may have different intended purposes. Medical devices that are to be sold in the EU need to have a Certificate of Conformity (CE marking). For dental implants, clinical testing has become mandatory to obtain the CE marking. In the United States, the FDA regulates a similar process in the so-called 501k procedure. In order to ensure similar testing standards for clinical studies all over the world and also to guarantee a maximum security for the patients involved in these studies, the Guidelines for good Clinical Practice GCP have been defined. [18] In studies that are intended to be used for the CE marking or for the 501k procedure, strict adherence to GCP is mandatory. This shows that proper planning and selection of the most appropriate study design becomes more and more important for the registration of a new medical device.

17.3.1. Parallel Group Studies

Study types can mainly be differed into descriptive, non-interventional studies and analytical, experimental studies that allow defined conclusions (Figure 17–2). A descriptive study explains “what happens in a population” regarding experience with an intervention, prevalence or incidence of disease. Case reports, case series or longitudinal single-armed studies are typical types of descriptive studies. These design types have limited value in the evidence hierarchy and are often reserved for very new techniques or the late phase after product registration (“Phase IV trails”). At this point, the researcher should distinguish between a case series, which is usually retrospective, and a cohort study, which is usually prospective. Unfortunately, the definition of the study type used is not always mentioned on the report of a study.

The prospective cohort study follows a study plan and can, therefore, not be retrospective. Typical open, non-interventional cohort studies have their strength in the external validity; a high number of patients without restrictive inclusion criteria might reveal specific side effects of a therapy.

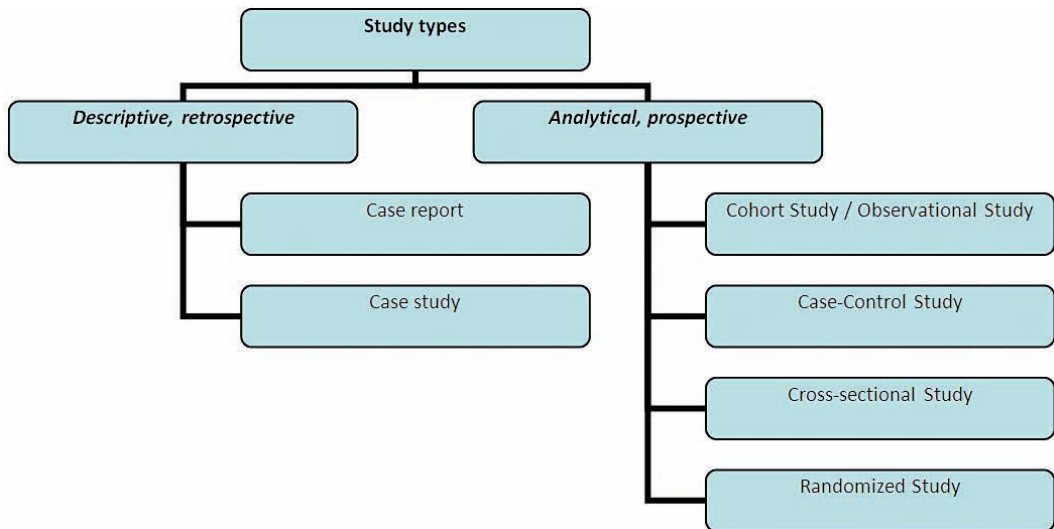


Figure 17.2. Clinical Study Types.

In medicinal products, Phase IV post-marketing trials are an important tool and can lead to a critical appraisal of a new drug. One interesting and often cited example were cardiovascular side effects of Cox-2 antagonists, which have been discovered by Phase IV studies after the market release of the drug (“Vioxx-scandal”). [19] In the world of medical devices, like dental implants, clinical studies before market approval are rare, and long-term data is missing in many new medical devices on the market. Once critical biological reactions to an implant system are seen in the clinic, it is usually reported as a case series or cohort study. [20] Therefore, data from these non-interventional studies can add important aspects to the understanding of the clinical properties of new implant systems or biomaterials. An often-critiqued aspect of large, industry sponsored Phase IV trials is that they might primarily be used as product marketing. So the researcher should always check for a clear scientific question behind these trials. Another requirement is that these studies should be registered and their results published irrespective of the results obtained. It is a well-known fact that studies with negative results are rarely published, and results from industry sponsored studies tend to be more positive in the interpretation (“publication bias”) [21].

Analytical studies, on the other hand, have a more complex design and help much better to quantify an effect of a certain intervention or therapy on a well-defined population. This requires a priori a clear study plan with definition of a (measurable) outcome and a control group. The idea of the parallel-arm clinical trial, using a control group, is attributed to the British physician Dr. James Lind. In 1747, many sailors, as part of his crew of a British naval ship, had been diagnosed by scurvy. He selected 12 ill sailors and divided them into groups of two with all similar symptoms. (“They all in general had putrid gums, the spots and lassitude,

with weakness of knees," he wrote in his 1753 paper, "A Treatise on the Scurvy"). The two sailors receiving oranges and lemons recovered from the symptoms, whereas the others were still ill, suffering of symptoms of scurvy. This parallel group design is still a basic principle in clinical trials but has the weakness that it is not assured that the participants in both groups really had similar symptoms of the disease; the treatment allocation might be biased.

17.3.2. Types of Bias

An important problem in clinical trial methodology is the systematic error ("bias"). These types of errors produce results that differ systematically from the truth. This is in contrast to random variation, which can simply be reduced by increasing the number of subjects in a study or experiment. Random variation (chance) leads to results that are imprecise, whereas systematic variation (bias) leads to results that are inaccurate. In implantology, this means that a huge observational study of early loading may produce results that are precise but not accurate due to the lack of randomization. In contrast a small, high-quality randomized controlled trial may produce results that are accurate but not precise. It explains why both study types are important to understand the nature of a therapy concept.

In planning the study, all efforts must be taken to identify possible types of bias and use methods to reduce them. Observational study designs are inherently more susceptible to bias than are experimental study designs. Broadly, three categories of bias are found: selection bias, measurement bias, and analysis bias. Selection bias describes the effect that the allocation of subjects into the groups might produce samples that are systematically different. A typical example is that risky therapy arms might not be applied in critical cases. For an early loading study, this could mean that implants with high primary stability are tended to be in the early loading group and implants with lesser primary stability in the control group. Random allocation into the study groups is the key to avoiding selection bias.

Measurement bias means that measurement of outcomes is inaccurate. This may be due to inaccuracy in the measurement instrument or bias in the expectations of study participants or researchers. Besides the use of objective rather than subjective outcome criteria, blinding of participants and researchers is one important step to reduce measurement bias.

The protection against bias created by allocation will only be maintained if all participants remain in the group to which they were allocated and complete follow-up. Participants who change groups, withdraw from the study or are lost to follow-up may be systematically different from those who complete the study. The so-called analysis bias can be reduced by adherence to follow-up and carrying out an analysis according to the initial group allocation ("intention to treat analysis"). In the example of an early loading study, patients in the test group with early loading might face more side effects and stop participating in the trial. This means that a patient, who is a "drop out," is from this point of view a very important one. He should be followed up and included in the intention to treat analysis. If this is not possible, at least the reason for the unwillingness to continue should be recorded.

17.3.3. Confounder

A confounder in a clinical study is a variable, which is related to both the risk factor (intervention) and the end point. This means that the risk factor (intervention) is not the only cause for the effect (end point); at least part of the effect can be explained by a confounder. This means that bias is related to measurement of a variable, and a confounder is related to the interpretation of a result. The most critical ones are unknown confounders. An often-reported example for a confounder is to interpret the finding that people who carry matches are more likely to develop lung cancer as evidence of an association between “carrying matches” and lung cancer. The important confounder in this setting is “smoking”—smokers are more likely to carry matches, and they are also more likely to develop lung cancer. For implantology, this means that if periodontitis, a possible risk factor for peri-implantitis and implant failure, is studied, smoking might be a confounding factor, as smoking might have a direct influence on the development of peri-implantitis. [22] A factor is not defined as a confounder if it lies on a direct pathway between the risk and the effect. In the peri-implantitis study named above, this would mean that bone loss at the teeth is not a confounding factor, as periodontitis causes bone loss, which again might be related to peri-implantitis.

Known confounders should be controlled by stratifying the groups accordingly; i.e., analyze smokers and non-smokers separately, or use statistical techniques to adjust for confounding. The role of unknown confounders is much more critical. There is always a risk that an apparent association between a risk factor, or an intervention, and an outcome is being attributed to an unknown confounder. This is a typical problem of observational studies where patients may be selected to treatment group or control group without an even distribution of unknown confounders. In a study on early loading in implantology study, this might mean that implants with lesser primary stability are found more in the control group than in the early loading group. The best strategy against unknown confounders is randomization, which helps that both known and unknown confounders are randomly distributed between treatment groups.

17.3.4. History of Randomization

Further development of the parallel group trial and the most powerful type of experimental study is the randomized controlled trial. The idea is that the allocation to the treatment groups is not done by the researcher or the patient but by an a priori specified allocation list. This is done to keep all things between groups similar except for the treatment of interest (principle of similarity). The first widely recognized randomized clinical trial was published in 1948, studying the role of streptomycin for the treatment of pulmonary tuberculosis. [23] The statistician Sir Hill is generally credited for the design of the trial. This trial can be seen as a landmark in a new era of medical research. Since that time, the randomized clinical trial has become a widely accepted standard in clinical study design, which is reflected by the exponential increase in number of these studies.

One of the most well-known studies, which has changed the way of thinking, is the CAST (Cardiac Arrhythmia Suppression Trial) Study, which was published in 1991 and 1992. The aim was to show the efficacy of class I antiarrhythmic drugs on heart-related death. In contrast with the suggested result, CAST I had to be stopped due to the fact that under

therapy with antiarrhythmic drugs, mortality was significantly higher than in the placebo group. [24] This impressive example is always cited to explain the pivotal role of a randomized controlled trial and shows that a clinically promising method might not prove to be superior to control. It is, therefore, hard to understand why this study type is so rarely used in dental implantology.

17.3.5. Blinding

Measurement bias can be controlled by first using hard, objective (“patient tangible”) outcome criteria. The second and even more important step is to blind (“mask”) all study participants to the allocation (study group) of a certain patient. The term of a double-blinded trial has been accepted, when both the physician and the patient are masked for the study allocation of the individuals. When planning a trial, the whole study team should be blinded, including monitor, data management and statistician. Unblinding is performed not before all analyses are done.

In a clinical setting, where therapy options are extremely different, blinding is often impossible. This is often the case when a surgical and a non-surgical method is compared (open flap surgery for peri-implantitis treatment versus conservative treatment) or while comparing different loading protocols in dental implantology. A good help in this situation is to involve a “blinded reader,” who is not aware of the study details. This can be done for measurement of radiologic bone loss or the interpretation of clinical photos using a score [25].

It is an interesting finding that in trials with subjective outcomes, the effect was exaggerated when there was a lack of blinding. [26] In contrast, in the same analysis, there was little evidence of bias in trials with objective outcomes for lack of blinding. This illustrates the important role of both objective outcome criteria and blinding.

17.3.6. Types of Treatment Allocation

To minimize especially the risk of selection bias, it is important to have similarity of patients in the test and control group. This can be achieved by random allocation of the patients to the treatment groups. It is obvious that there are methods of group allocation that seem to be randomization but have critical sources for possible bias: A typical example is randomization by birth date; it seems to be random, but the physician knows the group of the patients prior to the study inclusion. This can lead, in an early loading study, to the effect that patients with critical conditions depending on their treatment allocation are not included into the study. Thus, one important objective of randomization is that all study participants are not aware of the treatment allocation before inclusion of the patient. The randomization list has to be generated by a third independent person (Sequence Generation) and must not be available for the study participants (Allocation Concealment).

Another important aspect is that randomization has to be reproducible. Throw of dices does not allow reproduction and is therefore an inadequate method of randomization. In a clinical study, randomization has to be performed using an a priori generated randomization list. One of the aspects of rating the quality of a clinical study is the respective type of

treatment allocation. Far too often in manuscripts, the “patients were randomized” is found without further description of the method of treatment allocation [27].

In dental implantology, a critical point during the planning phase is that implant-wise randomization is often not practical. In an early loading study, a patient-wise randomization is more suitable. This can lead to an uneven distribution of known confounders between the groups (e.g., smokers)—a problem that is practically addressed using stratification. In multicenter trials, the center effect is balanced by producing a separate randomization list for each center. Known confounders are included into the randomization process and balanced.

17.3.7. Practical issues of Blinding and Randomization

In implantology studies, all efforts should be done to include blinding and randomization into the study design. Blinding can be either done using a code throughout the study (e.g., new, test bone substitute gets a neutral package with code A; control bone substitute is coded with B). This is a cost-effective way of blinding, which can only be done if the study products are really identical. Otherwise, blinding of the patient only might be applicable. The researcher should be aware that this efficient method of blinding (coding) can become critical if the study is unblinded before its end. Emergency envelopes have to be prepared for emergency situations. It is, therefore, crucial to inform all participants about the possible effect of unblinding one patient for the whole study blinding.

Randomization has to be done after all in- and exclusion criteria are checked. In many cases, randomization is done by fax or is web/PC based. Often in implantology, some criteria are assessed within the operation room (bone volume, primary stability). This means that randomization has to be done in the operation room during surgery; in these cases, prepared envelopes are still most suitable. In any case, the study team should discuss the practical issues of randomization in advance. The type and time point of randomization has to be fixed in the study plan and given in the study report or manuscript.

17.3.8. The Intention to Treat Principle

One very critical point in study conduction is the so-called “drop out” patient. This even starts before the inclusion of patients into the study: very restrictive studies excluding a high number of patients have reduced external validity. It means that the study result might not be representative any longer. Also, the possibility that patients with confounding variables have selectively been non-included is given. To overcome this, a patient screening log is mandatory. And when reporting study results, the reason for non-inclusion of a screened patient has to be given (Figure 17–3).

The even more critical problem is related to patients that are excluded after randomization. The balance that was achieved by randomization can get lost if patients are selectively lost for the analysis. [28] The intention to treat principle means that all patients are analyzed according to their initial treatment allocation, irrespective of the fact regarding which therapy they actually had. This is a conservative but safe approach to the problem of exclusion after randomization.

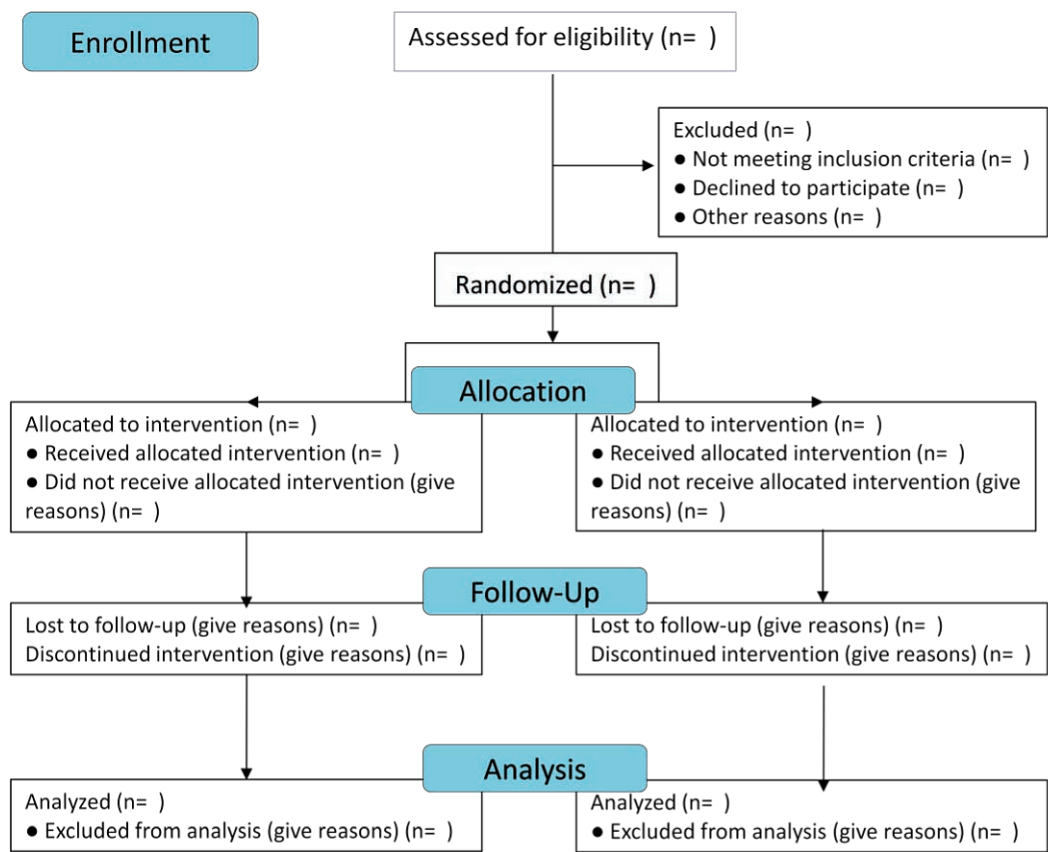


Figure 17.3. Flowchart of the patients in a clinical study according to the CONSORT statement.

17.3.9. Reporting of Randomized Studies

At has become obvious, the brand name “randomized controlled trial” itself does not alone stand for quality. The scientific community is aware that quality of reporting randomized controlled trials is getting more and more important but relies on the information contained in the publication. Important limitations of RCTs are insufficient power, poor reporting of randomization, inadequate randomization, failure in blinding, failure of follow-up. Thus, important efforts have been made to improve the quality of randomized controlled trials. In 1996, the "CONSORT (Consolidated Standards of Reporting Trials)" statement was published, which was revised last in 2010. [27] It is designed to assist the reporting of randomized controlled trials with two groups and those with parallel designs (Table 17–2, Figure 17–3).

Table 17.2. Checklist for the report/manuscript of a randomised clinical study according to the CONSORT statement

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomized trial in the title	
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	
	2b	Specific objectives or hypotheses	
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	
Participants	4a	Eligibility criteria for participants	
	4b	Settings and locations where the data were collected	
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	
	6b	Any changes to trial outcomes after the trial commenced, with reasons	
Sample size	7a	How sample size was determined	
	7b	When applicable, explanation of any interim analyses and stopping guidelines	
Randomization:			
Sequence generation	8a	Method used to generate the random allocation sequence	
	8b	Type of randomization; details of any restriction (such as blocking and block size)	
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	

Section/Topic	Item No	Checklist item	Reported on page No
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analyzed for the primary outcome	
	13b	For each group, losses and exclusions after randomization, together with reasons	
Recruitment	14a	Dates defining the periods of recruitment and follow-up	

	14b	Why the trial ended or was stopped	
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	
Numbers analyzed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	
Other information			
Registration	23	Registration number and name of trial registry	
Protocol	24	Where the full trial protocol can be accessed, if available	
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomized trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up-to-date references relevant to this checklist, see www.consort-statement.org.



CONSORT 2010 checklist of information to include when reporting a randomized trial*.

Some modifications will be required to report special trial designs including testing for non-inferiority. [29] Although the CONSORT statement was not evaluated before its publication, it was expected that it would lead to an improvement in the quality of reporting of randomized controlled trials, but still important information is not available on many cases. [30,31]

Especially for the preparation of systematic reviews, the adherence to the CONSORT criteria is of great value; [32] they are also a good help in the planning phase of a clinical study. Reviewers and editors of medical journals are strongly encouraged to ask authors of papers on clinical studies for adherence to the CONSORT criteria [33].

Many scientific journals require the registration of a clinical study. This is mandatory for drug-related studies but not yet for studies including medical devices. Registration of clinical studies can be done at official websites (clinicaltrials.gov) and helps to identify duplicate studies with similar questions.

17.3.10. Limits of Randomized Studies

Criticism has been raised that the focus on interpretation of the results of randomized controlled trials might have emphasized statistical significance rather than clinical importance. In some situations, statistically significant results may not be clinically important.

On the other hand, statistically insignificant results do not completely rule out the possibility of clinically important effects. [34,35] Some aspects also stand against the practicability of conducting an RCT.

This can be the case for a rare disease or if the therapy concepts are extremely different, including the necessity for sham operations. Another point is that RCTs need sponsoring, which is not available for all clinical questions. Cooperation with industry partners is a chance to conclude (expensive) high-quality RCTs. But it should also be kept in mind that conclusions in trials funded by for-profit organizations tend to be more positive (reporting bias) [21].

Conclusion

The challenge of matching a well-formed clinical research question to the most appropriate research method is simplified by an understanding of the various study designs available. Not all methods will produce useful answers in all cases, but each approach has its advantages and disadvantages; it is the knowledge and consideration of those disadvantages, not their elimination, that allows each of these methods to find its place in the spectrum of clinical research designs. It is obvious that adequate design of a clinical study is related to its chance for realization. It is neither rational nor possible to ask for an RCT in all clinical questions. [36] The study design should be planned to reduce as much risk of bias as possible and to control the important confounding factors.

In implantology, there is a chance for improvement of study quality starting from the planning phase to study conduction and reporting.

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Chapter XVIII

Inclusion and Exclusion Criteria in Clinical Research

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Abstract

Inclusion criteria are the parameters to admit a patient in a clinical trial, whereas exclusion criteria are the criteria for excluding patients from a study. The definition of inclusion and exclusion criteria represents one of the most important factors that permit researchers to correctly design and execute a clinical trial.

This chapter describes how inclusion and exclusion criteria should be selected when planning studies in the fields of implantology and tissue regeneration. It is important to realize that different clinical questions should be addressed by different study designs and, consequently, different decisions regarding inclusion and exclusion criteria should be taken in the planning phase.

18.1. Introduction

The choice of inclusion and exclusion criteria is critical when designing a clinical trial in all fields of medicine. These criteria, if correctly chosen, guarantee the selection of the appropriate study population for the trial's purposes, thus permitting reliability in the study results.

Inclusion criteria are the parameters to admit a patient in a clinical trial, while exclusion criteria are the criteria for excluding patients from a study. Ideally they have to be clear and objective, so that any investigator involved in the clinical trial can easily identify the patients that can be enrolled or excluded from the study during the screening visits. The inclusion criteria should also be clear and objective in order to allow other investigators that attempt to replicate the study, to reproduce patient inclusion decisions precisely. Our description of inclusion and exclusion criteria will be mainly focused on studies regarding implantology and tissue regeneration, a field in which it may be difficult to define clear and objective parameters when designing a study.

Some criteria can be easily defined and respected by the investigators: it is clear that it is not difficult to screen patients according to parameters such as their sex and their age and that confusions or mistakes in the enrollment very seldom arise. Other criteria can be much more difficult to be judged. As an example, several clinical studies in the field of implantology and tissue regeneration require patients with “good” or “adequate” or “high” oral hygiene levels. [1,2] These words may introduce some confusion, because what may be “adequate” for one clinician may be not “adequate” for another one. This may cause a problem when multiple investigators are involved in the same study, because different personal evaluations may cause a bias in the study results. In order to overcome this problem usually it is important to refer to objective scores or scales that can guarantee more standardized evaluations. Moreover in the studies where multiple investigators are involved, it may be beneficial to provide them some training and to organize one or several meetings in which the investigators try to find common and objective scales of evaluation. Usually in the first of these meetings the investigator who designed the study (generally called the “principal investigator”) describes the whole study and leads an open discussion with other colleagues in order to improve and eventually amend the protocol. Then, before the beginning of the study a “Calibration meeting” can be organized to discuss the measurements that should be taken at the screening visit to include or not include a patient in the experiment. [3] Very often patients not belonging to the experiment, or other healthy volunteers, are present at the calibration meeting to allow the investigators to perform a practical training and to verify the achievement of the homogeneity of evaluation among the researchers. A similar procedure is followed for each parameter of the selection that could be susceptible to inhomogeneous ratings and for the variables measured as study outcomes. Investigators may perform an inter-examiner reliability test that may be carried out before beginning patient enrollment and data recording [4].

The use of parameters that are not completely objective may also cause problems for the clinicians that read the study and try to replicate it or just try to apply the study results to their clinical practice. If a calibration is possible among the investigators of a clinical trial, it is clearly more difficult to reduce the problems related to the interpretation of unclear inclusion criteria made by clinicians who read the study after its publication and have no chance to discuss with the investigators. For this reason the consensus find in the investigators meeting on the measurement and the definition of the selection criteria should be widely described in the study so that the reader can determine if the samples studied correspond, or not, to the patients treated in daily clinical activity.

As previously mentioned, the different study designs satisfy different purposes. There is not a single research method that can answer all the clinical questions. Usually randomized

clinical trials are considered the best studies for questions related to therapy, while cross-sectional studies may answer questions related to diagnosis or screening.

Observational studies such as Case-control studies or Cohort studies are helpful when the question is related to the causative mechanism and Cohort studies are the best for prognosis. Observational studies are also very useful in identifying late or adverse events. [5] Such different study design calls for different criteria for the selection of the study samples and investigational procedures. In order to report the study protocols and results in the best possible way, some statements have been developed.

The aim of these statements is to provide the readers of a scientific paper with the possibility to deeply understand how the samples were selected, what has been done in the experiment and how the outcomes were measured and analyzed. This information is fundamental in order to be able to fully understand the conclusion of the study and in order to replicate the experiment.

The interventional studies are disciplined by the CONSORT STATEMENT. [6] This document entitled “Consolidated Standards of Reporting Trials” (CONSORT) was issued in 1996 and modified in 2001 and 2010 with the aim to improve the reporting of a randomized controlled trial (RCT), to allow the readers to understand a trial's design, conduct, analysis and interpretation, and to assess the validity of its results. The CONSORT STATEMENT emphasizes that this can only be achieved through complete transparency from authors [5,7,8].

The CONSORT STATEMENT set the minimum standards for writing a paper on a randomized clinical trial and improved the quality of reporting of RCTs [8,9].

A few years later a minimum set of recommendations for reporting has been defined for Observational studies. This is called the STROBE STATEMENT, where STROBE stands for “Strengthening the Reporting of Observational Studies in Epidemiology” [10,11].

18.2. Inclusion and Exclusion Criteria in Observational Studies

The distinction between inclusion and exclusion criteria is not always necessary and they can all be listed together as “eligibility criteria”. The most common inclusion and exclusion parameter criteria relate to age, gender, diagnosis and pathologic conditions, but there is evidence that they are very often not adequately reported in medical and dental literature [12,13].

For what concerns the inclusion and exclusion criteria in observational studies the STROBE CHECKLIST reports the following paragraph:

“6. POPULATION:

- a) Cohort study—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up.
 - Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls.

- Cross-sectional study—Give the eligibility criteria, and the sources and methods of selection of participants.
- b) Cohort study—For matched studies, give matching criteria and number of exposed and unexposed
- Case-control study—For matched studies, give matching criteria and the number of controls per case”.

The description of the population object of the study should be carefully detailed in order to help the reader understand if the conclusions may apply to the clinical problems he is dealing with. As in other types of studies, the samples are defined by demographic parameters, clinical conditions and other features of the patients. It is important to report on the group from which the participants of the study were chosen, such as ethnic origin and nationality, and the methods that were used to select the patients, such as advertisement or investigator’s proposal or others.

All the variables considered as eligibility criteria should be listed and widely described in the “materials and methods” section of the paper. The possibilities for a reader to completely understand which clinical situation has been tested during the experiment as well as the possibility of replicating the study are probably the most important issues that increase the scientific value of the paper [11].

For example, if patients with a particular disease are enrolled in one study group, it is not sufficient to generally report the presence of this pathological condition; precise diagnostic criteria should be presented. It may often be necessary to comment on the eligibility criteria in the discussion section in order to explain why that parameter was chosen in select patients and why that particular interval of measurement was used for screening.

The acceptance of the follow up protocol is another important issue among the eligibility criteria. The patients that accept enrollment into the study should know the follow up schedules and be able to accept it. This is important because it can minimize the number of lost to follow-up patients. The recall procedures as well as the methods to follow the participants should be described carefully and it should be pointed out if they are, or are not, equal for all the patients in all the study groups. This description adds value to the study and allows the reader to understand if the results could be affected by different follow up periods or recall protocols between the study groups or by inadequate follow up periods in relation to the studied variables [14].

In case-control trials, the selection of cases and controls is crucial. Different selection of cases and controls may result in different conclusions. The description of this selection should be extremely detailed in order to allow the reader to judge the validity of the trial. Patients belonging to the controls should reflect the same population from which the cases are selected [15].

Matched observational studies are the comparison between groups in which each participant belonging to the test group is matched by a comparable subject in terms of demographic data and other eligibility criteria. Case-control studies very often use matched groups. Matching is performed with the aim of obtaining homogeneous groups, so that the differences that eventually arise from the study can be related to the investigated variable. When groups are not matched there is a high chance of having inhomogeneous groups at

baseline and consequently a number of uncontrolled and confounding factors that may affect the outcome of the experiment and the study conclusions. [16,17] As an example, the clinical studies related to the implant field very often include patients with a wide age range (i.e. between 18 and 70). It may easily happen that one of the samples could be composed of significantly younger rather than older patients. This can be a confounding factor.

When matching is used, its rationale and how it is performed should be carefully stated in the methods section. Matching can be also used in cohort studies in order to achieve homogeneous groups at baseline, thus increasing the statistical precision of the study [11].

In the materials and methods section the authors should also clearly state when (in what phase of the clinical procedure) patients are assigned to test or control groups. If a group assignment is performed during the screening visit a bias is easily introduced since the clinician knows beforehand what variable will be included in the treatment or observation and may modify, without any specific will, the experiment because of personal considerations. The latest the group assignment is performed the less likely that a clinical bias may be introduced.

18.3. Inclusion and Exclusion Criteria in Interventional Studies

The CONSORT CHECKLIST reports the following paragraph for the inclusion and exclusion criteria in observational studies.

“4 – PARTICIPANTS: 4a - Eligibility criteria for participants. 4b - Settings and locations where the data were collected”.

As for the observational studies, the detailed description of the inclusion and exclusion criteria is crucial also for the interventional studies. It has been demonstrated in several studies that their description is often unsatisfactory or incomplete or sometimes absent. [18-20] The investigators and scientific writers must provide the reader with the chance to really understand the study and to replicate it. If the reader completely understands the study protocols, he may interpret the paper and define if the results are applicable to his case.

The applicability of the study conclusions are independent from different factors, and the eligibility criteria are certainly among the most important. Usually the interventional studies tend to isolate the studied variable, so as to avoid confounding factors that may influence the outcome. For this reason, generally, the eligibility criteria tend to be restrictive. This approach is very useful to identify if the studied variable modifies the outcome in the test group when compared to the controls. On the other hand the clinician that looks at the literature to find information in order to address his clinical question may often find these studies unsatisfactory, because they very precisely answer a very narrow question that is seldom exactly the same as the clinician's question. [21] The applicability of the study is also called “external validity” and refers to the possibility of generalizing the study conclusions to other situations [22].

Internal validity is related to the ability of a study to eliminate the possible bias through proper conduct. This is an issue that qualifies the study and its conclusions. It is clear that if a

study lacks internal validity, because its results are influenced by confounding factors, it also lacks external validity because unaffordable conclusions cannot be generalized. On the other hand it should be pointed out that the conclusions of a study that has proper internal validity cannot be applied to any clinically related questions. The absolute external validity doesn't exist.⁷ Very often the clinician has to interpret the papers because its "real world" questions may be different from the studies. As an example of this problem we can refer to a study that compares a machined smoothed vs. a sandblasted micro-rough implant surface, proving that the latter one guarantees a superior bone for implant contact without significant side effects and without a significant rate of implant failures. If a clinician has to decide whether or not he should use another sandblasted implant surface but with a different micro-roughness, he may refer to the mentioned paper and try to interpret it. He could speculate that the study conclusions cannot be applicable to his clinical situation, because he doesn't have proofs of safety and effectiveness of this third surface. He could also speculate that the use this surface can be safely performed because it is similar to the one tested in the study.

The key for this kind of judgment relies on the proper description of the eligibility criteria, of the setting and location of the study, of the clinical protocol, of the studied variables, of their methods of measurements and of the follow up procedures. It is important to report on the group from which the participants of the study were chosen such as ethnic origin and nationality and the methods that were used to select the patients such as advertisement or investigator's proposal or others.

The most widely used inclusion criteria in interventional studies are demographic information such as age and sex along with the definition of the pathologic condition that is analyzed in the study. The disease should be defined with objective parameters so that its stage and extent can be clear.

The release of informed consent by the patients is another fundamental inclusion criterion. It should be read, understood and signed by the patient and the clinician should be available to answer all the related patient's questions in order to clarify any aspect of his participation in the experiment. The informed consent form as well as the whole study protocol should be in accordance with local and international guidelines and should be approved by an Ethics Committee. The patients should accept the follow-up schedule and should be encouraged to be compliant at the recall visits. The investigator's issues to motivate patients should be exactly the same in both test and control groups.

Among the exclusion criteria there are usually pathologic conditions that can be worsened by the intervention.

As for the observational studies, there is not an absolute necessity for distinction inclusion and exclusion criteria and they may all be listed together as eligibility criteria.²³ Along with the eligibility criteria detailed information on the location of the study should be provided to the reader. Geographic, cultural, social, economic and environmental aspects could influence the generalizability of the study. It is important to describe the setting in which the study was performed (hospital, private practice or others) and if the study was multicentric or not [24].

The moment of the clinical procedure when patients are assigned to the test or control groups should be stated because, as already discussed for the Observational Studies, the possibility of introducing a bias may be influenced by the timing.

Interventional studies are generally used to identify the effectiveness of a certain therapy or medical device. When approaching the protocol design of an Interventional Study, the

researchers tend to isolate the influence of the studied treatment or device on the outcome of the therapy from other possible influencing factors. This approach may be very effective in determining the effectiveness of the studied therapy or device in narrow clinical situations, but may be not very useful when the study conclusions have to be generalized and applied to a wider range of situations that a clinician has to face. In order to avoid this problem the researchers may be induced to select broader inclusion criteria so that a wider range of situations may be included. This approach leads to other problems such as the fact that different factors may influence the results and that it may be difficult, if not impossible, to understand which of them plays a role on the study outcome and how.

On the basis of these considerations it is evident that a single study design cannot be exhaustive. It has already been stated that different study designs answer different clinical questions. The clinician should understand that it is not possible to solve all the questions related to a treatment or a medical device with a single experiment and that it may be necessary to perform several different Interventional Studies that focus on selected narrow clinical situations. Afterwards it may be useful to perform one or more Observational Studies on a wide range of clinical situations in order to identify the possibility of late or adverse events.

As an example, it may happen in the field of implantology that, after all the preliminary animal, preclinical and clinical safety studies, a new product, such as a growth factor for bone augmentation, comes to its market release. The researchers in charge of the clinical testing should plan to perform different Interventional Studies in order to verify the effectiveness of the new product in narrow clinical situations, such as its use in fenestrations around implants, in horizontal augmentations, in vertical augmentations, in sinus lift surgery, etc. These studies aim to compare the new product with others previously considered as the “gold standard” in each of these narrow clinical situations so as to identify and enlarge, if possible, the clinical indications for the newly introduced growth factor.

The same researchers usually plan one or more observational studies with larger samples and broader inclusion criteria in order to discover possible late or adverse events. It is evident that this is a big effort in terms of financial and human resources and that this requires a long time, but this is probably the conduct necessary to perform correct research, with reasonable confidence that possible errors be limited.

18.4. Examples of Eligibility Criteria Used in the Fields of Implantology and Regenerative Dentistry

18.4.1. Inclusion and Exclusion Criteria for a Randomized Clinical Trial Comparing Transmucosal vs. Submerged Healing of Implants Placed into Fresh Extraction Sockets [4]

Eligibility Criteria

Patients must have read and understood the written patient information paper and have acknowledged this by signing the patient informed consent form before entering the study.

Patients should be informed of the follow-up schedule that should be respected in order to guarantee good maintenance of the implant-supported restoration and in order to collect the study data. The patients must agree on this schedule and release their written agreement to attend the scheduled visits over the follow up period. Nevertheless the patients have the right to withdraw from the study at any time without prejudice. In this case a valid alternative treatment related to their dental condition will be offered by the investigators.

In order to be enrolled in the study the patients must comply with all of the inclusion criteria and will be excluded if one or more of the exclusion criteria are found.

Inclusion Criteria

- Males and females
- At least 18 years of age and not more than 70 years old.
- An immediate post extractive implant procedure (type 1) is foreseen in the maxillary canine and premolar post extractive sites and in the mandibular incisor, canine and premolar post extractive sites.
- Horizontal defect distance ≤ 2 mm
- Patients must be committed to the study.
- Patients must be healthy at time of surgery.

Exclusion criteria

- Medical conditions requiring prolonged use of steroids
- Hemophilia, bleeding disorders or coumadin therapy
- History of neoplastic disease requiring the use of chemotherapy
- History of radiation therapy to the head and neck
- Patients with history of renal failure or chronic renal diseases
- Patients affected by chronic liver diseases
- Patients with severe or uncontrolled metabolic bone disorders
- Uncontrolled endocrine disorders (including diabetes)
- Current pregnancy at the time of recruitment
- Physical handicaps that would interfere with the ability to perform adequate oral hygiene
- Use of any investigational drug or device within the 90 day period immediately prior to implant surgery on study day 0.
- Alcoholism or chronic drug abuse
- Immunocompromised patients including patients infected with HIV
- Patients who smoke more than 10 cigarettes per day or cigar equivalents, or who chew tobacco
- Conditions or circumstances, in the opinion of the investigator, which could represent a general contra-indication for surgical procedure or would prevent completion of study participation or interfere with analysis of study results, such as history of non-compliance, or unreliability
- Local inflammation, including untreated periodontitis to residual dentition
- Presence of fistula near the treatment sites
- Presence of an implant in one adjacent site

- Presence of vertical bone defects deeper than 2 mm around the implant shoulder positioned 2 mm below the CEJ of adjacent teeth
- Impossibility to obtain a primary stability
- Mucosal diseases such as erosive lichen planus
- History of local radiation therapy
- Presence of oral lesions (such as ulceration, malignancy)
- Severe bruxism or clenching habits
- Patients with inadequate oral hygiene or unmotivated for adequate home care

Comments

Since the intention was to compare the effectiveness of two healing patterns for implants placed into fresh extraction sockets, the authors decided to select cases of implants placed without the use of grafting materials. The use of grafting materials could have influenced the study outcome. From the literature available at the moment of the study design, there was evidence that in cases where the Horizontal Defect Distance ≤ 2 mm (horizontal distance from the implant to the socket internal walls) no augmentation was needed. For this reason it was decided to include in the study only patients with $HDD \leq 2$ mm. However this parameter could not be measured at the screening visit, but only during the surgery, after implant insertion. It was decided that if, at the moment of placement, the horizontal distance between the implant and the bony walls of the socket (HDD) was ≤ 2 mm, the patients should not be randomized. Consequently these patients should be included in the category 'intend to treat' and should not be evaluated for the study purposes.

Among the inclusion criteria the commitment to the study is crucial. This is a fundamental criterion in order to limit the lost to follow up cases. The problem in these cases is how to judge if the patients are really committed to the study, since it is not a measurable variable. The release of a written informed consent in which the follow up protocol is clear could certainly be helpful for these purposes, but is not definitive, since patients are allowed to withdraw from the study at any moment by current laws. Thus it is important to spend time with the patient with motivational speeches in order to maintain his compliance.

The exclusion criteria mainly deal with systemic pathologies or drugs that could interfere with tissue healing, including smoking > 10 cigs per day. Local factors such as uncontrolled infections, unhealed extraction sockets were listed as well as bad habits (such as bruxism) and the inadequate oral hygiene. All these factors could influence the outcome of implant healing and of its performance after loading and were correctly excluded.

The eligibility criteria in this study were correctly defined, but there were, as in any study, some limitations. Synthetically it may be affirmed that this study was designed to enroll healthy adults that need a post-extraction implant intervention of moderate difficulty ($HDD \leq 2$ mm and vertical defect ≤ 2 mm). The authors judged that it might have been difficult to find a reasonable number of cases with these very narrow bony features around the post-extraction implants. For this reason the study had a multicenter design and patients of almost all possible ages (age range 18-70) were accepted. One of the limitations of this study was that eligibility criteria did not include the matching of the study groups. Thus a group composed of patients significantly younger than the other could occur. Matching is performed "naturally" when the number of enrolled patients grows, but when the number of cases is

limited (as it very often happens in clinical studies related to implant dentistry) the need to match the groups should be carefully evaluated with the help of a statistical consultant.

18.4.2. Inclusion and Exclusion Criteria for a Split Mouth Randomized Clinical Trial Comparing different Techniques for Root Coverage

Eligibility Criteria

- Age range 18-60
- Good general health conditions
- No contraindications for periodontal surgery
- Smoking limited to 10 or less cigarettes per day.
- Presence of at least two adjacent buccal gingival recession defects affecting symmetric teeth on both sides of the upper jaw.
- Only Miller's Class I and II recession type defects with minimum length of 2 mm were included
- Only recession affecting upper incisors, canine and premolars were included
- Each group of multiple recession defects should be comparable in size with the contralateral defects: the mean recession length of one group should not differ more than 2 mm from the mean recession length of the contralateral group in the same patient.
- Presence of a clearly identifiable CEJ line
- Absence of cervical abrasion
- Absence of restorations on the root surface
- Full Mouth Plaque Score < 20%
- Full Mouth Bleeding Score < 20%
- Probing Depth $\leq$ 3 mm

Comments

The eligibility criteria were very well designed in relation to the definitions of local conditions of the treated recessions. It was clearly stated that only upper teeth in the anterior zone could be treated and the inclusion criteria admitted only multiple bilateral recessions on symmetrical teeth, in patients with a good level of oral hygiene and no periodontitis. Some criteria were also stated in order to include recessions of comparable gravity on the two sides.

This being a split mouth study, one side was assigned to the test group and the other side to the controls. This design, combined with the well-defined inclusion and exclusion criteria increased the value of the experiment because the selection of cases was restricted to very narrow clinical conditions and because both test and control surgical techniques were applied to each single patient, thus avoiding the potential bias related to individual responses. For the same reason the broad age range of inclusion was not a major problem in this study since all the patients receive both treatments and the study groups were matched for this parameter.

Eligibility criteria related to general health conditions could have been better described.

The most evident problem in this study setting is related to the extreme difficulty in finding a sufficient number of cases with these very narrow clinical conditions. A multicenter design could be helpful in these cases.

18.4.3. Inclusion and Exclusion Criteria for a Retrospective Observational Study Evaluating the Clinical Results of Mandibular Incisor Replacements with Narrow Implants [25]

Inclusion Criteria

- Patients treated with implants for one or more mandibular incisors replacement
- Patients treated between 2001 and 2003
- Patients treated with narrow implants (diameter $\leq 3,5$ mm)

Exclusion Criteria

- Multiple-unit restorations if cuspid or bicuspid teeth were part of the rehabilitation
- Patients that demonstrated inadequate levels of oral hygiene during the follow up period.
- Heavy Smoking habits (more than 10 cigarettes per day)
- Uncontrolled Diabetes or endocrine or metabolic disorders.
- Uncontrolled Periodontitis

Comments

It is evident that the list of eligibility criteria is reduced if compared to interventional studies. Many of the local or systemic exclusion criteria related to possible risks during surgery were not listed, because the surgery was already performed. The clinicians in charge of the treatment were supposed to have performed a proper anamnestic examination before the surgery.

The main conditions that could have influenced the treatment outcome, such as inadequate levels of oral hygiene, heavy smoking habits, uncontrolled diabetes or periodontitis, should have been recorded and excluded. It is difficult to find information in retrospective studies. These studies very often select only few eligibility criteria, thus including a wide range of treatment indications and being close to the clinical reality of the practitioners.

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Chapter XIX

Qualitative and Quantitative Methods for Evaluation of Dental Implant Osseointegration in Humans

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ABSTRACT

The clinical utilization of dental implants has accelerated in recent years, and new applications continue to emerge. The rapid development of various products and introduction of different treatment protocols have expanded the areas of application. As a result, an increase in the number of complications related to oral implants in humans can be expected. Research on techniques to solve compromised situations before implant treatment and complications and destruction of peri-implant tissues demands further development of sophisticated techniques to reveal the causal and modifying factors in these processes. The aim of this chapter is to describe the main methods available to monitor the biological performance of a biomaterial in the human body.

Introduction

Clinical trials constitute a prerequisite for the development of new and improved therapeutical tools in medicine. As described elsewhere in this book, all scientific clinical testing must be preceded by proper preclinical evaluations to demonstrate biological biocompatibility and efficacy. Clinical trials must comply with adopted ethical guidelines and follow Good Clinical Practice standards as described in the International Conference on Harmonization guidelines for good clinical practice (ICH). [1,2] These guidelines are intended to provide a unified standard for the European Union, Japan, and the United States to facilitate the mutual acceptance of clinical data by regulatory authorities in these jurisdictions. Compliance with this standard provides public assurance that the rights, safety and well-being of trial subjects are protected; consistent with the principles that have their origin in the Declaration of Helsinki 1964 and subsequent revisions [3], and that the clinical trial data are credible. All biomaterials and devices must develop objective safety guidelines for the device and indirectly, for the safety of the biomaterial used in the device. In order to be approved by governmental authorities in Europe and the US all data on performance of a material must be followed by statistically valid evidence. Often, indirect or non-invasive methods such as radiographs may prove inadequate to demonstrate biomaterial performance. In addition to strict scientific concerns, a clinical study also must provide the best care to each patient. In clinical research evidence based approaches have now become established as a mandatory foundation for appropriate protocols. This approach includes all aspects of the trial: informed consent, sample size determination / power analysis, strict inclusion/exclusion criteria, blind examinations, randomization procedure and, proper statistical methods to get to the best possible evidence level as described in the Consolidated Standards of Reporting Trials (CONSORT) statement [4]. However, several incidents of scientific misconduct of clinical trials for new products launched by dental implant companies exist and have caused widespread concern within the dental community [5]. The reason for this scientific misconduct can be due to the pressure to publish in order to get funding, personal ambition, or direct financial gain.

It is strongly recommended that professional statistical proficiency be included in the preparation of clinical research protocol in order to set appropriate parameters and to determine a sample size with sufficient power. However, it is beyond the scope of this chapter to discuss such aspects in detail and the reader is referred to other chapters in this book and to other literature.

To study the healing-in of biomaterials in general, and osseointegration of oral implants in particular, various study designs may be performed — it may be a proof of principle study to assess the safety of a new device or a study to evaluate the efficacy of a device or it may be a treatment approach. It can also be an efficiency study to validate findings in a previous efficacy study. Additionally, the study can be performed evaluating the effectiveness of a material, device or technique in the hands of clinicians.

Various parameters may be adopted in the clinical evaluations of implants. This chapter describes some methods available to monitor the biological performance of a biomaterial in the human body. These can be arranged in two parts — clinical parameters and parameters that include laboratory processing.

The clinical evaluation of an oral implant is traditionally focused on the healing-in of a material. The rapid introduction of various products and the steeply increasing number of installed implants has revealed an increasing bulk of complications related to oral implants placed in humans. Therefore, research on complications and destruction of peri-implant tissues demands further development of sophisticated techniques to reveal causal and modifying factors in these processes.

Clinical Assessments for Monitoring Peri-Implant Conditions

19.1. Per- and Postoperative Surgical Assessments

During placement of implants, the surgeon traditionally estimates the bone quality and quantity of the recipient sites, for example, according to the criteria set by Lekholm and Zarb [6]. It has been shown that this estimation is related to other criteria such as insertion torque values, RFA, etc. [7] However, some authors have shown that it is difficult to surgically discriminate between type 3 and type 4 bones. [8] Various attempts have been suggested to assess the quality of the bone and its volume in more detail. Some authors have measured the bone configurations at each implant site; the thickness and height, in order to test correlation with bone reactions during healing-in. [9] Also in peri-implantitis studies, the configuration of the defect has been shown by some authors to influence the result of treatment of the disease. [10] During surgery the primary stability of the implant can be assessed by insertion torque measurements which will be discussed below (19.2).

In addition, classifications have been introduced to assess the soft tissue wound healing over time. For example, Wachtel et al. [11] proposed a wound healing index that can be used in a modified form as suggested by Göthberg et al. [9] in implant studies as follows: 1: complete flap closure – no fibrin line in incision line; 2: complete flap closure – fine fibrin line in incision line; 3: complete flap closure – fibrin clot in incision line; 4: incomplete flap closure – partial necrosis in incision line; 5: incomplete flap closure – complete necrosis in incision line.

19.2. Implant Stability

19.2.1. Insertion Torque

Implant insertion torque has been shown to be a valid parameter to assess the quality of the recipient bone [12], and measuring the removal torque has been used to assess the extent of osseointegration for conventional implants. [13].

In numerous clinical investigations the primary stability of the implant is reported with implant insertion torque values e.g. [14-17] Several studies have shown correlation with insertion torque value (Ncm) to the presence of complications such as bone loss or implant failure due to compression of the bone resulting in necrosis. [18] However, the interpretation of data may be difficult because of the interaction of variables influencing the healing [19] and also because it is a one-time measurement. Also, questions have been raised as to whether

comparisons with different implant systems or designs using insertion torque values are possible at all. [20, 21].

19.2.2 Removal Torque

Removal torque force measurement has been widely used by a number of authors in order to find the relationship between the implant surface and bone in experimental animal models. [22-25] However, this procedure is a destructive method, leading to irreversible plastic deformation within peri-implant bone. It is necessary to remove an implant to measure the removal torque value. For this reason, human studies of removal torque for conventional implants are very rare. One such study reported the removal of 9 non-loaded titanium craniofacial implants from the mastoid process of human volunteers. [26] Only two reported human intraoral removal torque studies to date, the first one was performed to measure the removal torque of 2 non-loaded conventional implants, [27] and the second one to evaluate the removal torque force of loaded transitional implants in human subjects. [28] Further, increased research interest in the area of titanium mini-implants as anchors for orthodontic appliances has also played a valuable role in clinical evaluation of removal torque of osseointegrated implants in human. [29].

19.2.3 Periotest

In the daily clinic, the implant stability is most commonly assessed by percussion although more objective stability tests are available. The Periotest [30, 31] is an instrument measuring damping characteristics and originally developed to assess tooth mobility. The instrument measures the contact time and is recorded as Periotest values (PTV's). The instrument has also been used to assess implant stability. Studies have shown a linear correlation of contact time and implant stability. [32, 33] The sensitivity of the Periotest has however, been shown to be highly dependent on the positioning of the device to the implant abutment. In addition, no correlation was found with bone quality assessment. [34] The prognostic value of Periotest measurements on implant integration/loss is not established at present. [34].

19.2.4 Resonance Frequency Analysis

Meredith introduced this device [33] as a non-invasive tool to measure implant stability using Resonance frequency analysis (RFA). By exciting a transducer attached to the implant with a frequency, a resonance is recorded indicating the stability of the implant. [34, 35] RFA has been thoroughly studied and validated to removal torque testing in *in vitro* and animal models. [36-41] In addition, clinical reports have demonstrated the benefits of this technique especially in compromised implant cases or when immediate or early implant loading is performed. [15, 42] However, single readings using RFA (or Periotest) are of limited clinical value. The prognostic value of both the RFA and Periotest techniques in predicting loss of implant stability has yet to be established in prospective clinical studies. [34] (Please see Chapter XXI for more information).

19.3. Radiographic Assessment

Follow-up evaluations with intra-oral radiographs are used in most studies to determine marginal bone changes at implants. Moreover, intraoral radiography is regarded as a standard procedure in the evaluation of oral implants [43] and has been shown to correlate with clinical parameters. [44] Animal studies on correlation of radiography with histomorphometry have, however, showed contrasting results, [45-47] in concordance with periodontal studies. For example, Tonetti and co-workers [48] have shown that radiographic assessment slightly underestimates the intrasurgical defect level. It has been stressed that a proper technique with correct and reproducible projection geometry is mandatory. [49] The probability of predicting clinical implant instability from a radiographic examination can be low in populations with a low prevalence of implants showing clinical instability despite the relatively good diagnostic accuracy. [50] Modern digital techniques have further optimized radiographic evaluations. However, optimal light and monitor brightness and contrast settings must be ensured. [51]

There are several studies investigating the accuracy and precision of digital panoramic radiographs and most of the reports concluded that the digital panoramic system was equally as useful as conventional film-based panoramic radiographs. [52, 53] However, it was also reported that panoramic radiography had limitations of distortion, superimposition with variability in inter-examiner agreement. [54] Digital subtraction techniques have also been introduced to study bone changes around implants. [55-57] In addition, new tomographic modalities as Cone Beam Computed Tomography (CBCT) have been shown to be a superior tool for three-dimensional evaluation of tissue changes around implants. However, it is beyond the scope of this chapter to describe this in detail. The reader is referred to another chapter in this book.

19.4. Oral Hygiene Assessment

Formation and development of microbial biofilms on oral implants is an important etiological factor to the pathogenesis of peri-implant disease and has been correlated with the presence of clinically detectable plaque. [58] Monitoring the presence and development of plaque around implants was first proposed by Mombelli and co-workers [59] who used an index, (modified from the Silness L  e index [60] developed to monitor dental plaque formation) as follows: Score 0: No detection of plaque. Score 1: Plaque only recognized by running a probe across the smooth marginal surface of the implant. Score 3: Plaque can be seen by the naked eye (visible plaque). Score 4: Abundance of soft matter. Lindquist et al. [61] used a three-score index as follows: No visible plaque, local plaque accumulation, and general plaque accumulation greater than 25%.

19.5. Mucosal Bleeding

As a result of peri-implant infection redness and swelling along with bleeding of the peri-implant mucosa may develop (peri-implant mucositis). Similar to monitoring of plaque formation, Mombelli et al. [59] proposed a Sulcular bleeding index (modified from L  e [62] representing peri-implant mucositis as follows: Score 0: No bleeding when a periodontal probe is passed along the mucosal margin adjacent to the implant. Score 1: Isolated bleeding spots visible.

Score 3: Blood forming a confluent red line on margin. Score 3: Heavy or profuse bleeding. Apse and co-workers [63] proposed a similar 4-score index; 0=normal mucosa, 1=minimal inflammation with color change and minor edema; 3=severe inflammation with redness, edema, ulceration, and spontaneous bleeding without probing. It has been shown, however, that mucosal conditions have a weak correlation with changes in implant crestal bone levels. [64].

19.6. Peri-Implant Pocket Probing

Under healthy conditions, the depth of the peri-implant soft tissue crevice is around 2-4 mm, although higher values can be found in areas where the implant is intentionally placed deeper. Preclinical studies have shown that healthy sites do not bleed upon probing. [65] Inflammatory changes in the tissue will transform the peri-implant sulcus to a pocket that may bleed on probing.

19.6.1. Bleeding on Probing

Bleeding on probing (BoP) in the peri-implant sulcus is used to assess inflammatory changes in the peri-implant tissues and has recently been recommended to be performed periodically to monitor peri-implant soft tissue conditions. [66] Animal studies have shown a clear correlation with the absence of BoP and healthy conditions and reversely, presence of BoP and peri-implant mucositis or peri-implantitis. [65] However, some clinical studies could not find such correlations. [67] This may be attributed to different probing forces used in different studies. Other investigations have shown high negative predictive values of absence of BoP indicating stable peri-implant conditions. [68] Moreover, one study showed higher accuracy of BoP assessment at implant sites than tooth sites. [69] A more strict definition of various degrees of bleeding may improve this parameter. A recent study has proposed a qualitative definition of BoP as follows: 0=no bleeding, 1=minute (slight) bleeding from the pocket, 2= abundant bleeding. [9] It should be mentioned, however, that studies must be carried out using concomitant histology and biomolecular analyses must be carried out to validate such approach.

In addition to BoP, suppuration reflecting high numbers of PMN cells has been shown to be associated with severe peri-implant inflammation and tissue breakdown. [70-72].

19.6.2. Probing Pocket Depth

Implant macro-design most likely influences the possibility of peri-implant probing. In addition, probing may be impossible due to the design of the suprastructure. In implant studies, it is therefore strongly recommended to remove the suprastructure prior to probing (Figure 19-1).

Further, the extent of probe penetration is influenced by probing force and angulation, the diameter of the probe tip, the inflammatory status and the firmness of the soft tissue. [58] Probing depth measurements at implants and teeth may not be fully comparable due to structural differences of the tissues. Animal studies have shown that a probe extends closer to the marginal bone at an implant site than at the tooth. [58, 73].

Schou and co-workers, in an animal study, showed that there was no difference between teeth and implants under normal healthy conditions [74]. In contrast, under pathologic conditions probing at implants was significantly deeper than at the teeth. In a clinical study, Mombelli et al. showed that peri-implant pocket probing is more sensitive than periodontal pocket probing to force variation. [75].

19.6.3. Clinical Attachment Level

The clinical attachment level (CAL) is used in periodontal treatment to monitor changes of periodontal tissue support. Using a periodontal probe, CAL is measured from a fixed point of the tooth to the bottom of the periodontal pocket. In periodontal studies, this measurement has been shown to correlate with radiographic measurements and direct surgical assessments. [48] CAL has also been used in implant research. Quirynen and co-workers found that CAL was found on average 1.4mm coronal to the bone crest as assessed in radiographs. [76] Brägger and co-workers found CAL on average 1.17mm discrepant to radiographic assessment. [77] Conversely, a recent study, assessing clinical and radiographic changes in peri-implant soft and hard tissue levels in 150 Brånemark implants from 3 months to 1 year postoperatively could not show correlation between changes in CAL and radiographically assessed marginal bone level changes. [9] The different results in the cited studies may be related to the above described difficulties in probing the tissue in a standardized manner. In the latter study, the probing was performed around each implant after the suprastructure had been removed with the probe placed in an axial position. Also, the clinical measurements were done by one calibrated examiner.

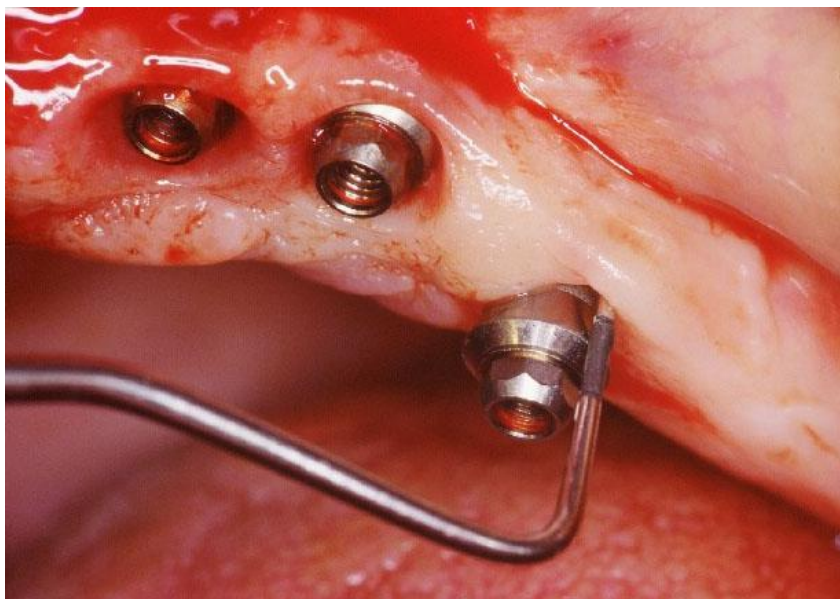


Figure 19.1. Clinical probing of peri-implant tissues after removing the suprastructure.

Laboratory Assessments

19.7. Microbiological Sampling

Recently, an excellent systematic review was published on this issue by Mombelli and Decaillet [78] to which the reader is referred for a deeper introduction. Parts of the text below are a compilation of this review. Clinical microbiological studies on oral implants have historically used culture techniques, [67, 79-81] direct phase-contrast microscopy, [67, 71, 81] transmission electron microscopy [82] or saliva samples. [83] Plaque samples were usually collected from the subgingival area using sterile endodontic paper points (Figure 19-2). Healthy peri-implant crevices have been reported to contain a predominantly coccoid microbiota while sites with deeper pockets exhibit significantly lower proportions of coccoid cells and a higher proportion of spirochaetes.

Colonization with potentially pathogenic microorganisms such as *S. aureus*, *Pseudomonas* sp. and enterobacteria has been reported. [82-84] *Fusobacterium* sp. and *P. intermedia* have regularly been found at diseased peri-implant sites while successful implants have yielded low cultivable counts and predominately Gram-positive cocci. In the darkfield microscope, samples from failing implants showed abundant motile rods, fusiform bacteria and spirochaetes, while samples from successful implants contained only a small number of coccoid cells and very few rods. [85].

Becker and co-workers used a DNA checkerboard PCR technique to study peri-implant microbiota. [86] They investigated the presence of some periodontal marker organisms and reported high levels of *P. gingivalis* or *P. intermedia* in patients with unsuccessful blade implants. Shibli et al., [87] using the same technique, found higher mean counts of *P. gingivalis*, *T. denticola* and *Tannerella forsythia* in a peri-implantitis group, both supra- and subgingivally. Marked differences were observed in the composition of supra- and subgingival biofilm between healthy and diseased implants. The microbiota associated with peri-implantitis was comprised of more periodontal pathogenic bacterial species, including the supragingival biofilm.

Early reports pointed to a microbiological similarity between peri-implant disease and chronic periodontitis. Over time, additional reports have suggested the possibility that some cases may show a different microbiological profile, similar to the microbiota generally associated with infections of implanted medical devices. In an interesting study, however not in the field of dental implantology, on molecular fingerprinting of *S. aureus*, efforts were carried out to determine if a clonal complex (CC) of *S. aureus* or certain virulence and adhesion factors were associated with infections of bones and prosthetic implants. [88] One hundred and nineteen isolates were characterized using microarrays. There was no evidence of a single virulence factor or CC being causative for bone and implant infections. Isolates belonged to 20 different CCs. Analysis of nasal swabs from 23 patients with infections of total knee or hip prosthetics showed that 65% carried *S. aureus*. In 39%, isolates from the infection site were identical to carriage isolates. An elevated risk of infection for *S. aureus* carriers was suggested and there was the possibility of endogenous infection in a high proportion of them.

Recently, array techniques have been demonstrated (see also below). In a recent paper, the microbiota in subgingival samples at peri-implantitis, periodontitis and healthy tooth sites

was analyzed using a 16S rRNA gene clone library. [89] Sequences were checked for artifacts and were thereafter aligned with a software program. A phylogenetic tree was constructed and libraries were analyzed with other software. A total of 77 different species were identified around implants.

The microflora was dominated by Gram-negative species and the diversity was found more complex around implants than at healthy or periodontitis sites. The 16S rRNA gene was amplified using PCR. *P. micra*, *P. stomatis*, *P. alactolyticus* and, additionally, *Chloroflexa tenericutes* and *synergistets* were found only in peri-implantitis sites. The authors suggested that the differences between peri-implantitis and periodontitis sites depend on different characteristics of the surfaces to which the bacteria adhere. In contrast to older studies the prevalence of putative periopathogens were found in low number at peri-implantitis sites.



Figure 19.2. Supragingival plaque was gently removed, and sub-gingival plaque samples were then collected using sterile endodontic paper points.

Beneficial effects of mechanical and chemical interventions to disrupt the peri-implant biofilm demonstrate that microorganisms are involved in the disease process. However, this is not proof that they are always the origin of the condition. Nevertheless, microbiological testing in combination with recording of BoP to monitor peri-implant tissue conditions long-term has been shown to enhance the prognostic value of BoP alone. [69].

19.8. Crevicular Fluid Analysis

The crevicular fluid around teeth and implants represents an inflammatory exudate containing a mixture of substances derived from serum, leukocytes, structural cells of the periodontium and oral bacteria. [90] Cellulose paper strips have been designed to collect crevicular fluid for further analysis of crevicular exudate (Figure 19–3). Various techniques have been used to analyze volume changes and specific molecular activities in the peri-

implant crevicular fluid (PICF) as well as in the gingival crevicular fluid (GCF). The quantity of exudate increases with the severity of the gingival inflammation [91] and, it has been assumed that the more severe the inflammation, the greater the dilution of the cytokines and MMPs. [92] It has, however, been reported that the concentration of enzymes is positively associated with the volume of exudate. [93] Villela et al. suggested that crevicular fluid volume and collagenolytic activity are two distinct and different parameters, although both are in some fashion related to inflammation. [94]

In PICF, on the protein level; enzyme-linked immunosorbent assays (ELISA) are the most commonly used technique. [95-100] PICF is collected by means of cellulose strips gently inserted into the peri-implant sulcus. The material is left in the sulcus 30-60s and thereafter transferred for further analysis. A wide variety of biomolecules have been studied, for example cytokines (IL-1 α , IL-1 β , TNF- α , IL-8), matrix metalloproteinases (MMP-1, MMP-3, MMP-8, MMP-9), and tissue inhibitor of metalloproteinases (TIMP-1). Periocheck[®], a colorimetric technique assessing neutral proteolytic enzyme (NPE) activity), [68] immunoblotting, [101-103] radioimmunoassay, [104] and spectrophotometric techniques [105] have been used. On the gene expression level semi-quantitative RT-PCR has been used. [106].

Quantitative Polymerase Chain Reaction (qPCR) represents a promising new tool to analyze and quantify spatially and temporally the biological processes in bone at a high level of precision and accuracy. [107] It has been used in many *in vitro* studies in relation to bone cells e.g., [108-111] and also in recent *in vivo* studies. [12-115].

In a recent study cellulose paper strips were found feasible to collect cells in the crevicular area of dental implants. [116] As revealed by light microscopic and SEM analyses leukocytes, erythrocytes and thrombocytes were detected at the surface of the strips. Further, qPCR was examined as a non-invasive diagnostic tool for the monitoring of healing-specific and peri-implant disease specific genes as a complement to clinical evaluations. In 18 partially edentulous patients, taking part in a prospective trial on various loading protocols of dental implants [9] crevicular fluid was collected using standardized paper strips (Periopaper[™], Proflow, Amityville, NY) at implants abutments from each patient 2 days, 14 days, 28 days and 90 days after surgery. Healing caps and FDP's were removed and cotton rolls were applied to avoid saliva contamination in the sampling area. One strip at each site was inserted in the crevice for one minute and thereafter placed in RNALater (Ambion, Applied Biosystems, Austin, Texas).

At the two days postoperative sampling, a considerable flow of PICF was seen at most sites. Gradually, this flow was reduced at the following sampling time-points. At day 90 hardly any fluid could be clinically detected. Carrier RNA was used to minimize losses of RNA during extraction. RNA was converted to cDNA. Quantitative PCR assays for IL-1 β , TNF- α , Osteocalcin (OC), Alkaline Phosphatase (ALP), Cathepsin K (CK), TRAP and 18S ribosomal RNA were designed and validated. Fluorescence detection was performed in the FAM/SYBR channel in the 72^oC step. Experiments were performed on the Light Cycler[®] 480 (Roche, Penzberg, Germany). After amplification a dissociation/melting curve was generated to verify that specific products were generated. Relative gene expression levels were calculated by normalizing gene expression of each gene using 18S ribosomal RNA and the $\Delta\Delta C_t$ method.

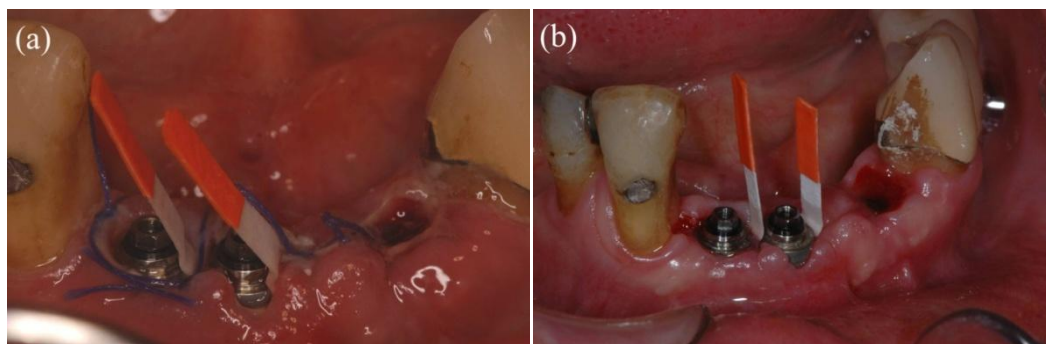


Figure 19.3. Cellulose paper strips after insertion in a peri-implant crevice to collect crevicular fluid in the same patient 2 days (a) and 2 weeks (b) postoperative. Note highly reduced volume of exudate in (b) compared to (a).

In general, gene expression for IL-1 β , TNF- α and ALP were found much higher than for OC, CK and TRAP. Initially, TNF- α was highly expressed and thereafter gradually decreased while OC and ALP slightly increased over time. No clear picture of the spatial changes was found for IL-1 β , CK, and TRAP. A significantly higher expression was found for IL-1 β at 2 days between the test and control group for smooth abutments. In one patient exhibiting unstable implants at 90 days, TNF- α was much higher expressed at 90 days than at the earlier assessments. Correlation was revealed with gene expression and clinical parameters at different time points. Of these, wound healing index [11], resonance frequency analysis, and clinical complication displayed more correlations with gene expression than the other parameters and showed frequent correlations with ALP, TNF- α and IL-1 β . ALP, TNF- α , and IL-1 β were most commonly correlated with clinical parameters. The strongest correlations were found for TNF- α at 2 and 14 days with clinical complication at 90 days. Hence, in the present study, some gene expressions even predicted complications. The amount of cells was however, mostly low at all collection time points. Besides this low amount of cells, gene expression was possible to detect by qPCR, confirming the high sensitivity of the method. [117-120] As a rule a larger amount of cells will generate a higher gene expression [121] however, as found by Slotte and co-workers [116], even extremely small cell samples can give rise to detectable gene expression. Also, the intensity of the specific expression and not only the number of cells are crucial for an accurate result. In general, a larger variation is gained with few target mRNA copies and it is the number of these copies that determines the result. To have a precise answer to this a fluorescence-activated cell sorting (FACS) analysis must be carried out exploring the relative amount of expression of *e.g.* TNF- α that can be found in an osteoclast. Further studies must be carried out to determine the precise detection limit for gene expression by this technique and should be followed by systematic studies mapping up- and down-regulation of genes during peri-implant wound healing and/or peri-implant tissue destruction. Also, further studies are needed to refine and optimize the sampling process, to find the appropriate panel and to validate gene expression for monitoring implant healing.

19.9. Biopsies

19.9.1. Histological Evaluation of Implants in Human Jawbone

Osseointegration has been defined as the direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant [122]. This definition has been described based on histologic evidence. Based on this definition osseointegration has been used as an index for prediction of long-term success of dental implants.

Histomorphometric and removal torque measurements are the most representative tests in studying the nature of the implant-tissue interface. [32] However, measurements of removal torque and percentage of bone implant contact require destruction of the study specimens; therefore, animal models have been extensively used for testing new products. However, data from experimental animal studies cannot be directly correlated to data from clinical human studies. Further, a variety of animal models have been used in dental implant research ranging from small laboratory animals to large animals. Use of small, mostly inbred, genetically homogenous laboratory animals like rodents or rabbits in experimental research has been shown to minimize biological variation in the results. [123] In contrast, biological reaction patterns in a human experimental population cannot be comparable to animal experiments due to great heterogeneity in the material regarding age, ethnic origin etc. In addition, the variation of anatomical implantation sites and observation times has raised a question about the validity and value of animal studies. Due to these great differences, unanimous support is established among scientists to the design and performance of thorough clinical studies before new products are implanted on a large scale in humans. [5, 124].

19.9.1.1. Retrievals

Histology and histomorphometry are very valuable in measuring the changes in the tissues that surround an implant. Various techniques (ground tissue-implant, decalcified, immunohistochemical sections) have been developed to evaluate the osteogenic potential of novel implant materials retrieved from the human jawbone (Please see Chapter XII for more information).

Histological examinations of clinically stable dental implants removed due to placement in a wrong position [125], or even in some cases, reports from postmortem [126] patients, have offered a unique opportunity to examine an osseointegrated human dental implant. Other less valuable histologic findings have been reported from clinically failed dental implants retrieved for various reasons, such as implant fracture, mobility, or peri-implantitis. [127].

19.9.1.2. Mini Implants in Humans

Small custom-made titanium micro-implants (2.5 mm in diameter and 5 mm in length) were designed for histological evaluation of new implant materials or implant surface modification (Figure 19–4). These micro-implants were usually placed in human jawbone during conventional dental implant surgery. [128, 129]

19.9.2. Soft Tissue Biopsies

Biopsies of human cases exhibiting peri-implantitis have shown high proportions of an inflammatory infiltrate [72] with high numbers of PMN cells. [71] Immunohistochemical

analyses have shown an intense inflammatory and immunologic response [70] and great proportion of B-cells and plasma cells. [130]

19.9.3. Gene Expression

Beside the above described qPCR technique, detecting expression of a panel of genes in an experiment, microarray techniques provide information on the expression of thousands of genes in a single experiment and in addition, make it possible to study expression patterns over time. Microarray analysis is often considered “discovery based” rather than “hypothesis driven”, because of the potential for discovering an altered expression of novel genes for which little or no prior basis suggests a role in the disease or for which the experimental condition examined has been considered. [131, 132] Limitations of this technique are the relatively high cost of each array and the handling of often huge amounts of generated data. However, increasingly sophisticated computational methods continue to be developed that are amenable to large data sets generated from microarray experiments. [133] As key disease pathways are identified, custom arrays containing relevant subsets of genes may become possible to integrate in clinical studies. [134] In skeletal research, animal studies have shown that more genes are upregulated than previously expected in later bone healing stages. [135] Recently, in two interesting experiments, Donos and co-workers studied gene profiling after implant placements in the mandible of human volunteers. [136, 137] Titanium mini-implants, with either a moderately rough (SLA) or a chemically modified moderately rough (SLActive) surface were placed in the retromolar area.

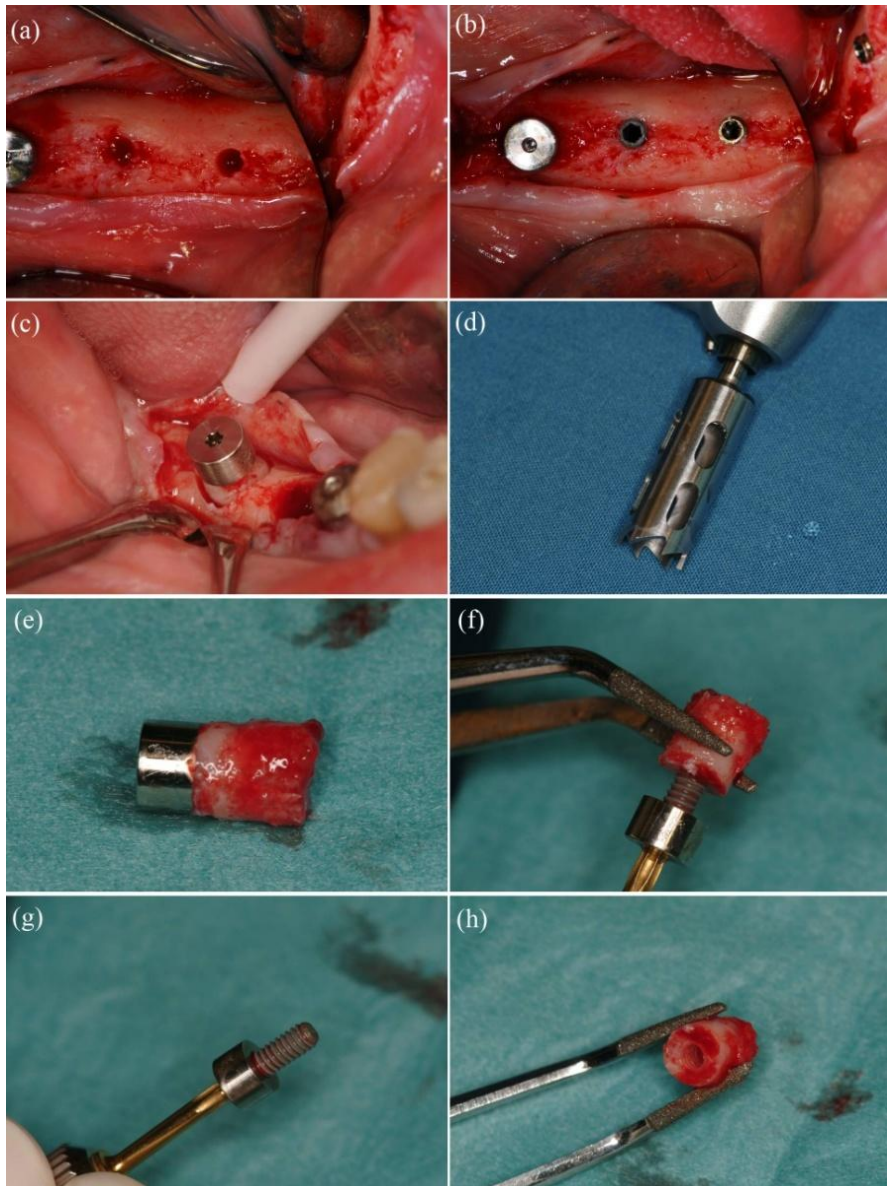


Figure 19.4. Retrieval procedure of micro-implants after the healing period. (a, b) Micro-implants were placed distal to conventional dental implant in lower posterior area. (c, d) A supra-abutment was used as a guide for the trephine. (e) Trephine mill with intact tissue collar and the screw-shaped micro-implants after explanation using trephine bur. (f, g, h) Unscrewing of the implant from the retrieved biopsy, and collected the bone samples and the unscrewed implants for gene expression analysis.

Biopsies containing implants and surrounding tissue were obtained after 4, 7 and 14 days of healing. The total RNA from the tissue was extracted and microarray analysis was carried out to identify the differences in the transcriptome between the SLA and SLActive surfaces. After 4 days, no difference in gene expression was found. At day 7, osteogenesis- and angiogenesis-associated gene expression was up-regulated on the SLActive surface. These expressions appeared to be regulated by BMP and VEGF signalling, respectively. At day 14, VEGF signalling remained upregulated on the SLActive surface, while BMP signalling was

upregulated on the SLA surface interpreted as a delayed compensatory response. The authors also found upregulated neurogenesis on both surfaces. Further, in the companion paper, Ivanovski and co-workers [137] studied the temporal gene expression profile associated with the early healing events during osseointegration using the same surgical protocol as described above. Nine mini-implants with an SLActive surface were placed and were removed after 4, 7 and 14 days postoperatively. Microarray analysis was carried out to identify the differences in the transcriptome between days 4, 7 and 14. At day 4, gene expression was associated with proliferation and immuno-inflammatory processes. After 14 days, the most predominant expression was associated with bone formation. In addition, angiogenesis and neurogenesis were also predominant by day 14. I κ B kinase/NF- κ B signalling was predominant at day 4, whereas TGF- β /BMP, Wnt and Notch signalling were all associated with the osteogenic process over the duration of the study. Furthermore, Ras and Rho protein signal transduction was regulated throughout the osseointegration process. (Please see Chapter XV for more information on molecular biology techniques).

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Chapter XX

Radiographic Techniques in Implant Research

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20.1. Introduction

Prosthetic rehabilitation by means of endosseous titanium implants, as an alternative to conventional prosthetics for replacement of congenitally missing or lost teeth, is a routine treatment modality. Radiographic examination is a basic indispensable component in all phases of dental implant therapy, including preoperative treatment planning, diagnosis, evaluation of treatment, and longitudinal monitoring of the results.

In general, uncontrolled use of radiographic examinations results in an unnecessarily increased radiation burden to patients and increased use of economic resources; obviously, it is important to identify indications, possibilities and, especially, limitations of radiographic examination. During the years, a variety of two-dimensional (2D) and 3D imaging techniques have been widely employed both in clinical and preclinical experimental settings in connection with the various phases of implant treatment. More recently, technological advances and decreasing costs have made 3D imaging techniques more accessible to general dental practitioners on a more widespread basis. Indeed, cone beam CT-scanning (CBCT) technology is currently being heavily marketed for cross-sectional imaging; nevertheless, it must be taken into account that this technique may be associated with an increased radiation dose [1-4] and higher costs compared with conventional tomography and panoramic imaging,

and that access to CBCT is still rather limited for the majority of dental practices. [5] In addition, this technology is currently primarily useful for diagnosis and implant treatment planning but not for monitoring of treatment, because of the extensive beam-hardening artifacts adjacent to the implant surface as a result of the metal. On the other hand, it is acknowledged that this technology will likely become the standard in the near future, as it is already in some parts of the world.

Taking the above in consideration, the current chapter will discuss some relevant aspects of 2D radiography, with focus on implant research. 3D radiographic techniques will only be shortly mentioned in perspective of 2D radiography. More specifically, the issues of a) reliability of radiographic measurements to estimate the true marginal peri-implant bone level, b) projection geometry for proper implant image quality and evaluation of the marginal peri-implant bone level, c) dimensional distortion in periapical and panoramic images, and d) timing of radiographic examination for evaluating the marginal peri-implant bone level during monitoring of implants will be discussed.

20.2. Panoramic and Periapical Radiography in Implant Research

All radiographic imaging techniques have strengths and weaknesses, and a combination of different methods may be needed to optimize the diagnostic outcome depending on the purpose. The most widely used radiographic examinations in association with implant treatment are panoramic and periapical imaging. Generally, panoramic radiography provides an overview of the jaws and an overall status of the teeth and their periodontal and periapical conditions, including the interrelations of the various anatomical structures. Panoramic radiographs are cheap and highly available, and the radiation dose of digital equipments is comparable to approximately four periapical radiographs. On the other hand, panoramic radiographs may show more artifacts due to projection of other non-dental anatomic structures (e.g. the spine), more overlapping among neighboring teeth – particularly premolars in the upper jaw –, and possess lower spatial resolution than periapical images; such issues may, for example, negatively influence measurement precision. In studies evaluating the periodontal bone level in panoramic or periapical images in comparison with direct intrasurgical measurements, panoramic images had a significantly lower accuracy than periapical images in terms of detecting bone loss and estimating the bone level (i.e. mostly underestimating the bone level). [6-8] Thus, panoramic radiography cannot be considered the method of choice for research purposes for evaluating details regarding the outcome of treatment or for monitoring the peri-implant bone level where measuring precision is required. Panoramic images are for the same reasons not considered appropriate for evaluating marginal peri-implant bone conditions in the clinic. Nevertheless, the image quality has improved during recent years, especially in the frontal regions, due to technically controlled, more penetrating radiation given to this region during the exposure that blurs the shadow of the spine in the image. Thus, the usefulness of panoramic radiography may increase in the future.

Panoramic examination has been used for evaluating the outcome of maxillary sinus augmentation procedures, probably because the entire sinus including the apical portion of the

implant can be more easily visualized in these images compared to the smaller-field periapical radiograph. [9,10] In general, panoramic radiography in relation to implant dentistry is customarily used for selecting the appropriate implant size and position during treatment planning in straightforward cases where the bucco-lingual dimension of the alveolar process can be clinically judged and provided that the distortion of the image is taken into consideration by a calibration procedure, as well as for obtaining an overview of the jaw bones and teeth.

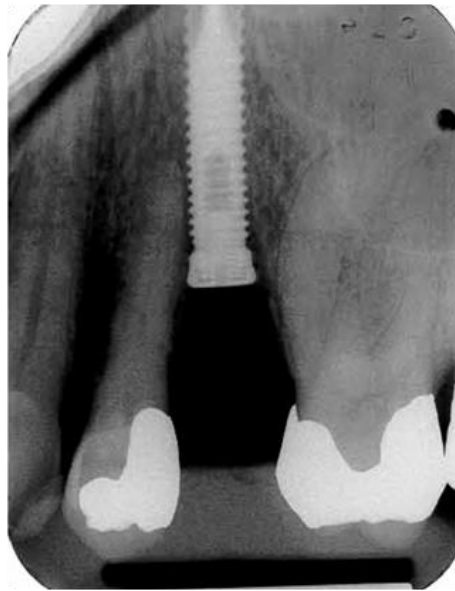


Figure 20.1. Due to extensive bone loss, the implant is located considerably more apically than the neighboring teeth; displaying the entire implant is difficult in a periapical radiograph.

In contrast to panoramic imaging, good quality intraoral periapical radiographs provide high spatial and contrast resolution and thus sharp images, but display only a limited part of the alveolar process and consequently may in some instances register only part of an implant. This is often the case in situations where extensive bone loss due to disease and/or physiological remodeling after long periods of edentulism has resulted in a low residual alveolar bone ridge in comparison with the neighboring alveolar crest (Figure 20–1). Periapical radiography in relation to implant dentistry is most useful in identifying the location of the immediate neighboring anatomical structures, including the roots of adjacent teeth, as well as local pathology, e.g. periapical lesions. Periapical radiography is also used for monitoring the outcome of implant treatment. Monitoring is important for controlling for possible biomechanical complications, e.g., component fracture, misfit between the implant and the abutment or between abutment and the margins of the prosthetic restoration, as well as for evaluating the peri-implant bone level for early detection of biological complications, thus allowing timely therapeutic interventions.

20.3. 3D Radiographic Techniques in Implant Research

An obvious disadvantage of both periapical and panoramic radiography is that only a two-dimensional image is displayed. The bucco-lingual dimension can be captured with cross-sectional radiography, such as conventional tomography, CT or CBCT. Indeed, conventional tomography, CT and CBCT have in most studies been found superior to intraoral and panoramic radiography with regard to visualizing anatomical structures, e.g. the mandibular canal. [11-20] On the other hand, in investigations comparing conventional tomography and CT rather diverging results have been obtained. In some studies CT performed better than conventional tomography as regards measurement accuracy or localization of anatomical structures, [16,21-23] while in other studies conventional tomography was superior or equal to CT. [13,17,24] Recently, Liang et al. [25] compared the image quality and visibility of anatomical structures in the mandible among five CBCT scanners and one Multi-Slice CT system (MSCT), and CBCT was found to be comparable or even superior to MSCT. Furthermore, 3D radiographic techniques (CT and CBCT) have been found superior to periapical radiography in identifying various types of artificially created bone defects (including periapical defects) [26-30] or bone lesions of endodontic origin [31,32].

Inherent disadvantages in currently available 3D volumetric radiography, however, seem to preclude their use for monitoring the peri-implant marginal bone level. The major disadvantage regards beam-hardening artifacts adjacent to the implant body or any other metal component of the implant restoration. These artifacts seriously affect the quality of the image at the implant-bone interface and in turn preclude obtaining reliable bone level measurements. [33] The beam-hardening artifacts within the reconstructed images are cupping and streak artifacts. Cupping artifacts occur when the multi-energetic radiation beam passes through a round or oval object with a high atomic number, e.g. metal. The softer parts of the radiation beam is absorbed in the object, and the part of the beam that has succeeded in penetrating the center of the object will be harder when it reaches the receptor than the part passing through the edges. Streak artifacts appear as dark bands between two dense objects, such as two dental implants in the same jaw. These artifacts have been previously shown to influence the visibility of both bone and soft tissues and result in inaccurate assessment of the peri-implant bone level. [34-36] In the study of Corpas et al., [34] despite the good correlation between the marginal peri-implant bone loss estimated with CBCT and that measured on histological slices, radiographic peri-implant bone level evaluated with CBCT was estimated on average 1.12 mm higher than the true histological bone level. Extensive efforts, for example based on mathematical modeling, are currently being made to minimize or avoid such artifacts. [37] Another disadvantage of the technique is the generally higher radiation dose delivered to the patient compared to that of the 2D techniques. [38,39] Due to those disadvantages 3D volumetric radiography has been mostly used for implant treatment planning when the clinical examination is insufficient, and not for evaluation of the treatment outcome.

20.4. Reliability of Radiographic Measurements to Estimate the True Marginal Peri-Implant Bone Level

Evaluation of implant treatment in the clinic includes visual inspection and assessment of mobility of the prosthetic restoration and the implant, assessment of oral hygiene, and registration of surrogate variables, including bleeding on probing (BOP) or suppuration and probing pocket depth (PD) measurements, which are used for detection of signs of disease. In contrast to teeth, however, where PD values compatible with healthy (stable) conditions are more or less accepted, the “physiologic” depth of the peri-implant sulcus/pocket is not defined. Implants are often “starting their life” with deep pockets (≥ 5 mm), for example due to anatomical factors, like when the alveolar crest at an edentulous site is located more apically than that of the neighboring sites where the teeth are present, or due to esthetic concerns that necessitate deeper (subcrestal) implant placement. PD around implants is thus sometimes difficult to interpret. In contrast to peri-implant mucositis that is a reversible inflammatory process in the soft tissues surrounding a functioning implant, peri-implantitis is an inflammatory process additionally characterized by loss of marginal peri-implant bone.⁴⁰ Knowledge of the marginal peri-implant bone level – and particularly of changes in bone level over time – is thus critical for evaluating pathology and establishing the correct diagnosis and consequently for considering treatment options. Evaluation of the marginal peri-implant bone level is also interesting in implant research when assessing the effect of various implant designs and surfaces on the physiological bone remodeling occurring after implant installation and loading. Thus, radiographic examination is essential in monitoring implant treatment to determine the marginal peri-implant bone level and assess possible changes (i.e. bone loss) between different time points.

A crude method of evaluating the peri-implant bone level in radiographs in cases with threaded implants is by counting the number of threads showing no bone-to-implant contact. However, in cases of cylindrical implants (no implant threads available) or for research purposes requiring data precision, peri-implant bone levels are evaluated by determining relevant reference landmarks in the radiograph and then measuring the distance from these landmarks to the most coronal radiographic bone-to-implant contact. It is obvious that the landmarks should have a fixed spatial position and be clearly and undoubtedly identified, if any meaningful conclusions regarding possible bone level changes at various examination intervals should be made. Usually, the shoulder of the implant is used as the fixed reference landmark, as it is most often readily recognizable; however, in cases of butt-joint connection, where sometimes identification of the fixture-abutment margin can be difficult, the margin of the prosthetic restoration or the apex of the implant can be used. For example, in Figure 20–2, the implant shoulder is quite easily identified in a case where the implant is equipped with a cover screw (i.e. before second stage surgery), whereas the same landmark may be difficult to recognize when the abutment and prosthetic restoration are mounted.

Table 20.1. Overview of studies using radiographic and histological/histomorphometrical evaluation

Study	Animal model	Related parameters	Evaluation period	Radiographic receptor	Comments
Abrahamsson et al., 1999 ⁴¹	Dog	Radiographic and histomorphometrical distance from first bone-to-implant contact to the top of the implant.	9 months	Conventional film	Both methods showed similar bone levels; the radiographic evaluation underestimated bone levels by <0.5 mm. No direct correlation was calculated.
Hermann et al., 2001 ⁴²	Dog	Radiographic and histomorphometrical distance from the first bone-to-implant contact to the top of the implant.	6 months	Conventional film	Both methods showed similar bone levels; the differences between the radiographic and histomorphometrical measurements were within 0.1 mm in >70% of the cases. A statistically significant correlation was found.
Gotfredsen et al., 2002 ⁴³	Dog	Radiographic and histomorphometrical distance from the first bone-to-implant contact to the top of the implant.	15 months	Conventional film	Both methods showed similar bone levels; the radiographic evaluation underestimated bone levels by <0.5 mm. No direct correlation was calculated.
Wikesjö et al., 2006 ⁴⁴	Dog	Radiographic bone formation level with the histomorphometrical bone regeneration height.	2 months	Conventional film	In general, the radiographic evaluation underestimated bone level compared with histomorphometry by <0.5 mm. No direct correlation was calculated.
Albouy et al., 2008 ⁴⁵ , 2009 ⁴⁶	Dog	Radiographic and histomorphometrical distance from the first bone-to-implant contact to the top of the implant.	5 months	Digital	Two papers reporting on the radiographic ⁴⁵ and histomorphometrical ⁴⁶ evaluation of the same material. In general, the radiographic evaluation underestimated bone level by 0.6 - 1.4 mm.
Duyck et al., 2011 ⁴⁷	Mini pig	Radiographic and histomorphometrical distance from the first bone-to-implant contact to the top of the implant.	3 months	Digital	Both methods showed similar bone levels; the radiographic evaluation underestimated bone levels by <0.5 mm. No direct correlation was calculated.
Choi et al., 2010 ⁴⁸	Dog	Radiographic and histomorphometrical distance from the first bone-to-implant contact to the top of the implant.	4 months	Digital	Both methods showed similar bone levels; the radiographic evaluation overestimated bone levels by <0.1 mm. No direct correlation was calculated.
Corpas et al., 2011 ³⁴	Mini pig	Radiographic and histomorphometrical marginal bone defect depth.	3 months	Digital	The radiographic evaluation underestimated the bone level by an average of 1.17 mm; in 50% of the cases underestimation was <0.5 mm. A statistically significant correlation was found.

A relevant question in this context seems to be: does recording of the marginal peri-implant bone level in periapical radiographs correspond to the true situation? In other words, how accurate are periapical radiographs in determining the true (histological) marginal peri-implant bone level? It is obvious that this issue can be basically explored only in animal studies; however, only few animal experiments involving clinical-type implants have directly addressed the question. Nevertheless, useful information can be obtained from studies reporting both radiographic and histological data. Table 20–1 presents an overview of relevant studies evaluating the marginal bone level around implants by means of radiographic and histomorphometrical parameters. [34,41–48] In general, all studies showed that both methods detected the same trend (i.e. when bone loss was detected histologically, the result of the radiographic analysis also revealed bone loss, and *vice versa*) but there was a tendency that radiography underestimated the bone level.

Two studies [34,42] found a significant linear correlation between the radiographic and the histomorphometrical parameters. More specifically, Hermann et al. (2001) [42] found a correlation of 0.993 ($p < 0.001$) between the two methods, and the differences regarding the distance from the implant shoulder to the first bone-to-implant contact as evaluated in periapical radiographs and in histological sections were within 0.1 mm in more than 70% of the cases. Similarly, in the other recently published study, Corpas et al. [34] observed a statistically significant correlation of 0.7 ($p < 0.01$) between bone level measurements made in digital intraoral radiographs and those in histological sections. When using a cut-off point of 1.5 mm for vertical bone loss, a sensitivity of 0.5 and a specificity of 0.71 were found for radiography compared to the gold standard. In addition, in this study radiographs underestimated the bone level by 1.17 mm on average; in 50% of the cases the differences were smaller than 0.5 mm. This means that radiography may indeed in some cases fail to diagnose a considerable amount of bone loss.

In a few studies (Table 20–2), PD measurements were made in addition to the radiographic and histological parameters. [49,50] In general, in both studies all methods detected the same trends for the various evaluated parameters. However, one of the studies [49] reported that peri-implant bone loss was estimated more accurately in periapical radiographs compared to clinical probing measurements irrespective the use of standardized or non-standardized pressure, while there was apparently no or only a weak correlation between the clinical and the radiographic recordings. The seemingly poor correlation between clinical and radiographic values can be explained by the fact that probe penetration within the peri-implant soft tissues is highly dependent on the level of tissue inflammation. [51] Specifically, even slight inflammation in the peri-implant tissues allows probe tip penetration close to the bone level.

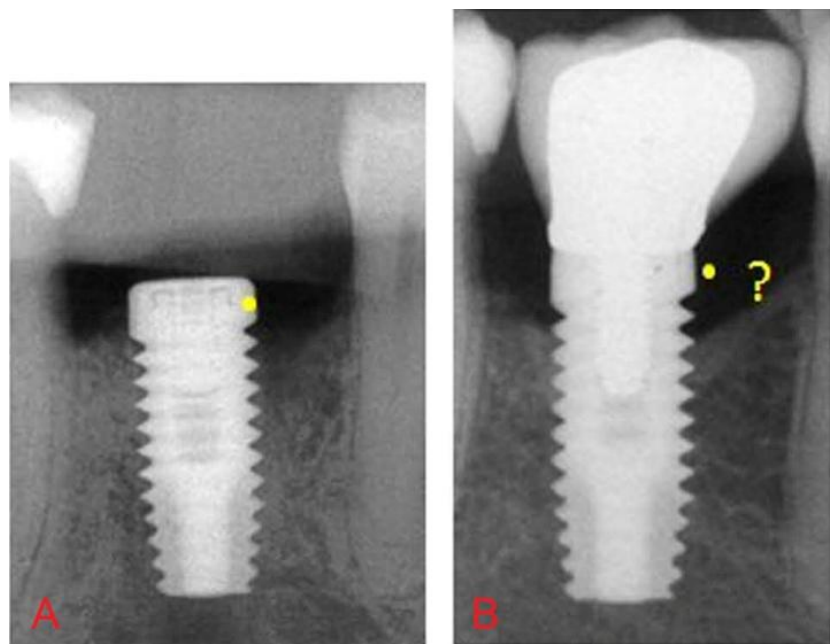


Figure 20.2. The implant shoulder is quite easily identified when the implant is equipped with a cover screw (A). The same landmark may be difficult to recognize when the abutment and prosthetic restoration are mounted (B).

Table 20.2. Overview of studies using radiographic, clinical and histological/histomorphometrical evaluation

Study	Animal model	Related parameters	Evaluation period	Radiographic receptor	Comments
Isidor, 1997 ⁴⁹	Monkey	Clinical measurements and radiographic and histomorphometrical distance from the first bone-to-implant contact to the top of the implant.	18 months	Conventional film	In general, radiographic assessment closely estimated the histomorphometrically determined marginal bone level, while probing in general failed to do so; the radiographic evaluation underestimated bone level by ≤ 0.6 mm. Direct correlation was calculated.
Zechner et al., 2004 ⁵⁰	Dog	Probing depth, radiographic bone loss height (in classes) and the histomorphometrical distance from the first bone-to-implant contact to the implant coating level.	4 months	Conventional film	Overall trend in the clinical and radiographic results agrees with histomorphometrical data. No direct correlation was calculated.

The periapical images in some of the above studies were obtained by conventional film radiography and in others by digital receptors, either sensors or photostimulable phosphor plate systems. No significant differences have been reported between conventional and digital periapical radiography regarding the diagnostic accuracy in detection and size estimation of

peri-implant defects. [52-56] Similar findings have been observed regarding periapical and periodontal defects, [30,31,57-61] while no differences seem to exist among the various intraoral digital systems [62,63].

Thus, the literature suggests that the marginal peri-implant bone level can be estimated more precisely in periapical radiographs than with clinical probing (with or without standardized probing force). In this context it is however important to mention that radiographic evidence of bone-to-implant contact does not necessarily imply osseointegration on the histological level [64].

20.5. Subtraction Radiography in Implant Research

An alternative method to simple linear measurements of the marginal peri-implant bone level in intraoral radiographs is subtraction radiography. Subtraction radiography is a well-established method for detection of bone changes in serial radiographs. The technique was introduced in the 1930s and has been in use during the 1990s in relation to various diagnostic tasks in dental research, e.g. detection of dental caries or root resorption, [65,66] healing effect after periodontal treatment, [67-69] temporomandibular joint function, [70] and assessment of morphologic changes and bone formation and remodeling of extraction sites. [71] Subtraction radiography has also been used in several studies for follow-up evaluation after implant treatment [72-76].

Several subtraction radiography techniques have been used, for example manual, semi- or fully automated systems. Some systems are based on the principle that a computer program simply calculates the pixel-pixel difference between two digital radiographs, while other systems are more advanced and, based on contrast adaption, subtract the gray shade value of each pixel in one image from the corresponding pixel value in another image, resulting in the subtraction image that represents the differences in gray shades between the pixels values in two radiographs.

If the two radiographs to be subtracted are recorded with standardized projection geometry and properly aligned, the differences in gray shades in the bone may be interpreted as differences in bone mineral content. For example, Reddy et al. [73] demonstrated a high repeatability of a semi-automated computer-assisted method used for measuring bone loss around implants. In earlier studies, subtraction radiography was used to detect not only changes of the marginal bone level around implants, but also for assessing bone remodeling, for instance assessing implant integration during the healing phase at the entire surface of the implant.

Brägger et al. [74] demonstrated peri-implant density changes during the early healing phase and during ligature-induced peri-implantitis in an animal model, as well as cases documenting loss of peri-implant bone density associated with infection or increase in density caused by remodeling after functional loading of an implant with a single crown. Recent studies evaluated also bone quality around implants based on this technique with variable results. [77-81].

Despite the fact that subtraction radiography has been advocated for detection of minor bone changes, one must be aware of the weaknesses associated with this technique. The major

limitation of subtraction radiography when analysing changes in a given bone defect is that the visualization depends on the bucco-oral width of this defect.

Total bone fill with uniform density in a bowl- or cone-shaped defect, as in an augmented peri-implantitis defect or an immediate implant placed in an extraction socket, respectively, may be visualized only in the coronal part. This is because the width of the defect at that point makes up a larger fraction of the total width of the alveolar bone in the coronal part as compared with the apical part.

Likewise, one must bear in mind that the subtraction image is a product of remodeling of the bone walls buccal and lingual/palatal to the defect on one hand and bone formation/resorption within the defect on the other; obviously it may not be possible to distinguish among these biological phenomena.

However, it must be pointed out that these limitations are not related to subtraction radiography in itself, but rather related to the two-dimensional nature of conventional radiography in general.

In perspective, due to the above shortcomings and the high costs associated with the software, the cumbersome training in use of the technique – and thus the markedly increased time for radiographic evaluation – as well as the fact that the new digital systems give better possibilities for direct visualization of shades of gray, subtraction radiography has not achieved widespread use in implant research.

20.6. Projection Geometry for Proper Image Quality and Evaluation of the Marginal Peri-Implant Bone Level

In order to facilitate proper assessment of the marginal peri-implant bone level, high-quality intraoral radiographs of proper density and contrast, and especially recorded with optimal projection is a prerequisite. Even small deviations from an optimal projection can lead to erroneous estimates of the peri-implant bone level. For example, when assessing a screw-type implant on a radiograph taken with an incorrect projection angle, i.e. the radiation beam was not perpendicular to the long axis of the implant, the threads appear blurred on either side of the implant, and it is impossible to accurately define the most coronal bone-to-implant contact (Figure 20–3). On the other hand, with an optimal projection angle in the vertical plane, which means that the film or digital receptor was positioned parallel to the implant, and the radiation beam was directed perpendicular to its long axis, the image displays sharp threads with no overlaps on either side of the implant. This facilitates proper evaluation and recording of the peri-implant bone condition (Figure 20–4). In this context, standardization of the projection angle seems also necessary to compare the bone level in radiographs of the same implant taken at different time points.

Obtaining optimal implant images may be challenging due to the fact that implants may not always be inserted with an inclination corresponding to that of the alveolar process or neighboring teeth. This might especially be the case in fully edentulous patients owing to the consequences of previous disease, but also in single tooth replacement in agenesis sites where variable amounts of alveolar bone may be missing. Correct alignment of the radiation beam

may also be difficult to predict while the implant is still submerged or before the prosthetic restoration has been mounted.



Figure 20.3. A radiograph taken with the radiation beam not perpendicular to the long axis of the implant results in an image with the threads appearing blurred on either side of the implant. This makes it difficult or impossible to accurately define the most coronal bone-to-implant contact.

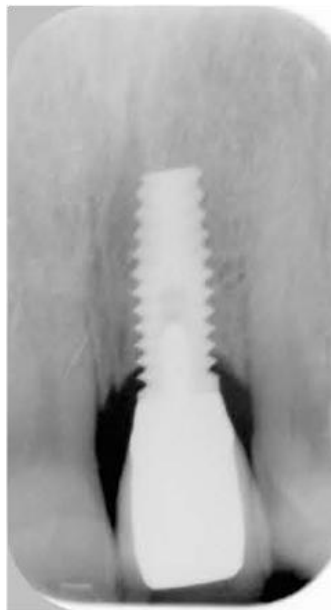


Figure 20.4. A radiograph taken with the radiation beam perpendicular to the long axis of the implant and the film or digital receptor positioned parallel to the implant results in an image appearing with sharp threads and no overlaps on either side of the implant. This facilitates proper evaluation of the peri-implant tissues and recording of the peri-implant marginal bone level.

Moreover, even after placement of the implant supra-structure, the implant axis cannot always be determined from a clinical examination because the abutment and restoration may be angulated in relation to the implant. As already mentioned, an image displaying sharp threads with no overlaps on both sides of the implant reflects an optimal projection in the vertical plane. On the other hand, blurred threads on either side indicate that the direction of the radiation beam had either an obtuse or an acute angle in relation to the implant. Since all screw-type implants are inserted by a clockwise turn of the screw, threads appearing mostly blurred on the left or right side of the implant indicate that the radiation angle was obtuse or acute, respectively, in relation to the long axis of the implant [82].

Based on this principle, a simple mnemonic rule can be applied in order to correct the inclination of the X-ray tube to obtain a second image with sharp implant threads. The rule may be memorized by a simple acronym, RB-RB/LB-LB. RB/RB: if Right Blur then Raise Beam (Figure 20–5). This means that if the implant threads are mostly blurred at the right side of the implant, the X-ray beam direction must be raised towards the ceiling to obtain sharp threads on both implant sides. LB/LB: if Left Blur then Lower Beam. This means that if the implant threads are mostly blurred at the left side of the implant, the X-ray beam direction must be lowered towards the floor to obtain sharp threads on both implant sides. Of course, the film must also be repositioned so that the radiation beam is kept at a right angle to the film/digital receptor; thus, use of a film-holder seems beneficial. Similarly, it is obvious that the position of patient's head needs to remain stable. The degree of radiation beam correction can be roughly estimated by looking at the degree of overlapping of the threads; of course, experience plays a role here. In general, for screw-type (threaded) implants, with a deviation of the radiation beam of up to about 10 degrees from the right angle to the axis of the implant, the threads on one side of the implant are still rather clearly discernible (Figure 20–6). [83] Deviation of the radiation beam of about 20 degrees from the right angle to the axis of the implant, means that the threads of both sides of the implant are poorly discernible (Figure 20–7). The RB-RB/LB-LB rule applies irrespective of whether the implant is placed in the upper or lower jaw. In a recent *in-vitro* study [84] evaluating the implementation of the RB-RB/LB-LB mnemonic rule, it was shown that even inexperienced operators (third-year dental students) could after a short instruction in the use of the rule record better quality implant images in two-thirds of the cases by changing the vertical projection angle in the correct direction from one exposure to the next. In this study, clinically acceptable images, either perfectly sharp or only slightly blurred threads, were obtained after an average of two exposures, even in the cases of extreme – compared to the neighboring teeth – implant inclinations.

Previous studies have presented various techniques for reproducible recording of implants with intraoral radiography. [85-90] Meijndert et al. [89] showed that an excellent reproducibility of hard as well as soft peri-implant tissue measurements could be achieved when using an acrylic device for the recording of dental radiographs and clinical photographs. Nevertheless, systematic evaluation of the possible advantage of using a projection standardization device instead of a free-hand method regarding the number of radiographs required for obtaining an acceptable image of an implant is limited. In a recent *in-vitro* study on plastic jaw models, Schropp et al. [85] evaluated the use of customized imaging guides (acrylic splints) against the RB-RB/LB-LB mnemonic rule with a standard film guide regarding the efficacy of obtaining perfectly sharp or clinically acceptable images, which meant only slightly blurred threads. The customized imaging guides were individually

prepared for each implant on the basis of a prefabricated dental X-ray film holder that consists of a horizontal occlusal plate and a vertical plate, housing the film (digital sensor herein). First, a 2 mm hole was prepared perpendicularly to the occlusal plate of the film holder with a bench-drill. Then, a long laboratory try-in rod was screwed into the implant, and the film holder was fitted to the rod through the hole on its occlusal plane; in this way the film holders' vertical plane was parallel to the implant axis. The film holder was then transformed to an acrylic splint by adding a light curing gel material in a thick layer onto the occlusal plate and the opposing occlusal surfaces of the model teeth (Figure 20–8). In this way, it was possible to place the customized imaging guide on the model in the correct position even after removal of the try-in screw. Construction of each customized splint took approximately 10–15 minutes. The results showed that the first exposure was rated as perfect or clinically acceptable in statistically significantly more cases with the customized imaging guide method than for the mnemonic rule method. However, after a maximum of two exposures no significant differences between the two methods were observed for achieving a perfect/clinically acceptable image (78% vs. 69%, respectively). Similarly, images with perfectly sharp threads in both sides of the implant with the first exposure were obtained significantly more frequently (4 times more) with the imaging guide method comparing to the mnemonic rule, but the difference was not significant after two exposures.

Taking into account the burden of constructing a customized imaging guide it would be reasonable to suggest that for standard monitoring purposes in the clinic or in cases of large field studies the RB-RB/LB-LB rule performs adequately; in cases of smaller-sized studies, where precision and high quality images are important, a customized imaging guide should be preferred. At this point, it must also be mentioned that use of a “rigid” system like the imaging guide method has some additional limitations.

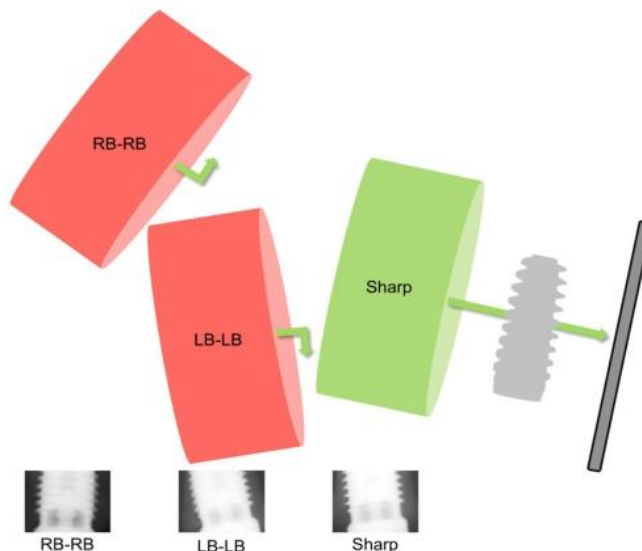


Figure 20.5. Graphic presentation of the RB-RB/LB-LB rule: If the implant threads are mostly blurred at the right side of the implant, the radiation beam direction must be raised towards the ceiling to obtain sharp threads on both implant sides. If the implant threads are mostly blurred at the left side of the implant, the radiation beam direction must be lowered towards the floor to obtain sharp threads on both implant sides.

For example, a precise and reproducible imaging guide position during consecutive examinations with several years interval might be problematic due to guide distortion or changes in the dentition, either physiologic or prosthetic in nature.

Also, it may be challenging to register the whole implant length, due to difficulties in proper film/sensor/plate placement as a result of the imaging guide shape and dimensions; however, this seems not to be a major problem since in most of the cases the area of interest is the marginal peri-implant tissue.

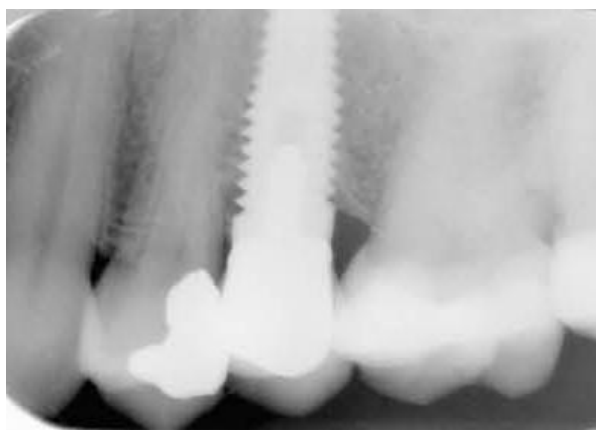


Figure 20.6. For screw type (threaded) implants with a deviation of the radiation beam of up to about 10 degrees from the right angle to the axis of the implant, the threads on one side of the implant are still rather clearly discernible.



Figure 20.7. For screw type (threaded) implants with a deviation of the radiation beam of about 20 degrees from the right angle to the axis of the implant, the threads of both sides of the implant are poorly discernible.

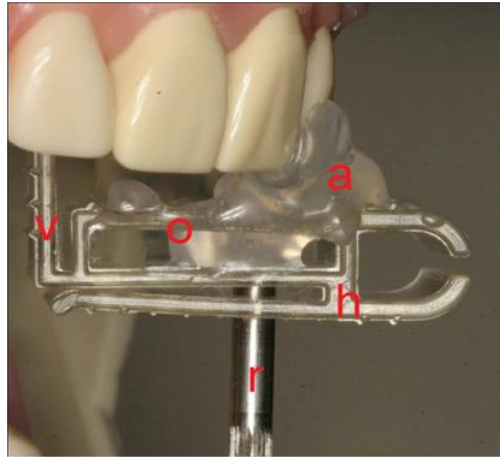


Figure 20.8. The film holder (h) is transformed to an acrylic splint by adding a light curing gel material (a) in a thick layer onto the occlusal plate and the opposing occlusal surfaces of the model teeth, while the film holder is fitted to a long laboratory try-in rod (r) through a hole drilled perpendicularly on its occlusal plane (o); in this way the film holders' vertical plane (v) was parallel to the implant axis.

20.7. Dimensional Distortion in Periapical and Panoramic Radiography

A drawback of both periapical and panoramic imaging is size distortion (enlargement and/or reduction) of the displayed structures, a well-known phenomenon in radiography. On average a rather large magnification is inherent in panoramic images compared with periapical radiographs, which moreover varies with region in the same image as well as in the horizontal and vertical plane. The magnification moreover depends on several factors, such as patient position, mandibular angulation and equipment. [91,92] In a recent textbook on dental radiography, it was stated that magnification in panoramic images varies between 10% and 30%; [93] nevertheless, a “standard” magnification factor of 25% is generally accepted. [94,95]. In clinical everyday practice, this known magnification is usually adjusted for when panoramic images are used for implant treatment planning. The simplest approach is to use a transparent sheet with drawings of the available implants (type, length and diameter) for a given system usually enlarged by the standard magnification factor of 25%. The sheet is placed on top of the radiographic film at the intended implant position, and an implant fitting the available bone site can thus be selected.

Although the use of such a crude method may be useful in the everyday practice, it cannot be acceptable for research purposes, where small differences may be critical for the outcome of the study or the interpretation of the results. In a previous analysis by Sonick et al., [19] measurements in panoramic radiographs were compared with direct measurements (by means of a caliper). Regarding estimation of the mandibular canal position, a variation in the magnification from 5% to 39% was observed; in true distances, this translated into 0.5 to 7.5 mm with an average difference of 3.0 mm. Similarly, in a recent study, [96] the true magnification in panoramic images was estimated by measuring the dimensions of a standardized metal ball of known size that was placed close to the anticipated implant site

during exposure; although the average magnification was 22%, a great variation in magnification ranging from -10% to 90% was observed. In concordance with previous findings, [92] it was also observed that the variation in magnification was larger in the horizontal plane than in the vertical plane (i.e. distortion), and the largest deviation from the standard magnification factor of 25% was seen in the horizontal plane and was most pronounced in the maxillary anterior region (Figure 20–9). [96] Based on the fact that large variation in panoramic image distortion exists depending on the region in the mouth as well as on the equipment it seems reasonable to suggest that a reference marker of known dimensions, for example a metal ball, should be placed on top of the alveolar process in the area of interest during exposure and used for adjustment to true size before measurement. [97] Moreover, when dealing with digital panoramic images the transparent sheet method will not work since the image “size” as displayed on the monitor depends both on the spatial resolution of the image and the line resolution of the monitor. In digital imaging calibration can be done by means of dedicated software that can read image information (e.g. resolution, true size, etc.) and includes implant templates in various sizes and brands.

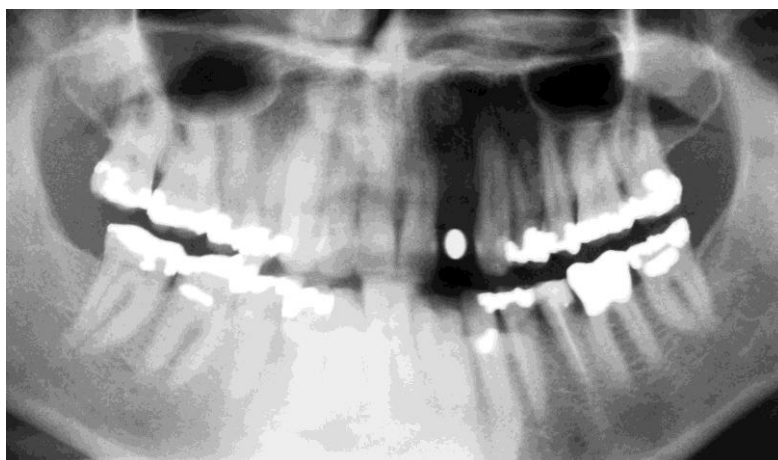


Figure 20.9. Distortion in panoramic images is usually larger in the anterior region of the mouth; in this image the metal ball appears as oval instead of as a circle.

Regarding periapical radiographs, image magnification varies with the relative focal spot-to-film and object-to-film distance; [98] in general, magnification is not considered a major problem in periapical radiography with not much variation among the various sites within the mouth, and an average magnification factor of 5% is accepted as the “standard” for radiographs recorded with the paralleling technique. [99] In the study by Sonick et al. [19] however, periapical images obtained by using a long-cone paralleling technique with a film holder attached to the tube of the X-ray unit showed a magnification ranging from 8% to 24% (14% on average). This magnification translated into a difference in absolute values between the periapical measurements and the true clinically measured distances ranging from 0.5 mm to 5.5 mm with an average difference of 1.9 mm. Similarly, Schropp et al. [96] observed an average magnification of 6%, however the range was from 1% to 12%. Distortion of such magnitude may not constitute a major problem in everyday clinical practice, but it is intolerable under research conditions. Thus, estimation of the magnification is imperative also

in periapical radiography, and the use of the “standard” factor of 5% seems not adequate for such purpose.

Several methods have been suggested for calibration purposes, for example the use of cylindrical metal markers or gutta-percha markers [100] or a metal ball with standardized diameter. [96] Gutta-percha markers seem not convenient because they cannot be easily sterilized while autoclavable cylinder markers, in addition to calibration purposes, can be used as indicators for implant angulation. Although no information on implant angulation can be obtained with a metal ball, this method has the advantage that the radiographic image of a metal ball is not influenced by projection geometry due to the symmetrical shape of the sphere. The possible influence, however, of the geometrical shape of a given marker on the accuracy of calibration is yet to be studied. In periapical post-surgical implant images it may not be necessary in most cases to include a reference marker. Instead, the implant itself with known dimensions or the known distance between the peaks of the implant threads (in cases that the entire implant is not captured in the radiograph) can be used for calibration purposes. Of course, this latter method is only possible to use in cases where radiographs with an optimal projection angle in the vertical plane, i.e. the image displays sharp threads with no overlaps on either side of the implant, are available (see previous section). In addition, it is obvious, that in cases where pre-surgical radiographs are to be included in the analysis, a reference marker should also be included.

20.8. Timing of Radiographic Examination for Evaluating the Marginal Peri-Implant Bone Level during Implant Monitoring

A critical issue when monitoring implants by radiography is the time for follow-ups. It has been suggested that in longitudinal clinical research, radiographs should be obtained at baseline (i.e. after insertion or prosthesis delivery) and at 1-, 3-, and 5-year intervals; thereafter, radiographs should be obtained every 5 years if marginal peri-implant bone level stability has been observed. [101] As mentioned earlier, differential diagnosis between peri-implantitis and peri-implant mucositis is based on the presence or absence of ongoing bone resorption. On the other hand, early marginal peri-implant bone loss of varying magnitude is a common finding regardless the implant type, although some implant designs (e.g. two-piece external connection) may result in larger amounts of bone loss compared to others (e.g. two-piece internal connection). Suggested reasons for this bone loss, which is generally concluded within the first year of function, are among others the surgical trauma, the presence of a fixture-abutment micro-gap, and the inevitable formation of a biological width with certain dimensions; [102] indeed, much effort is currently put into avoiding or minimizing the marginal peri-implant bone loss. The magnitude of bone loss varies among implant designs, but in cases of two-piece implants with a but-joint connection, the bone level after one year of implant function is often located 1.5 to 2 mm below the implant-abutment junction. [103] It becomes thus obvious that in such cases, using a radiograph taken immediately after implant installation or prosthetic restoration as the baseline image to be compared with images obtained several years later might lead to erroneous conclusions regarding the presence of

peri-implantitis (Figure 20–10). In this context it is interesting to mention that in most of the studies evaluating the outcome of implant therapy in periodontitis susceptible patients in comparison with that in non-susceptible individuals, immediately post-surgical or post-loading images were used to represent the baseline status.

This, in turn, may question the significantly increased peri-implantitis rate in periodontitis susceptible individuals reported in those studies. In order to avoid this pitfall and/or in the absence of proper baseline images, i.e. after one year of restoration, one may use a larger than 1.5-2.0 mm marginal peri-implant bone loss as cut-off point for defining a peri-implantitis case.

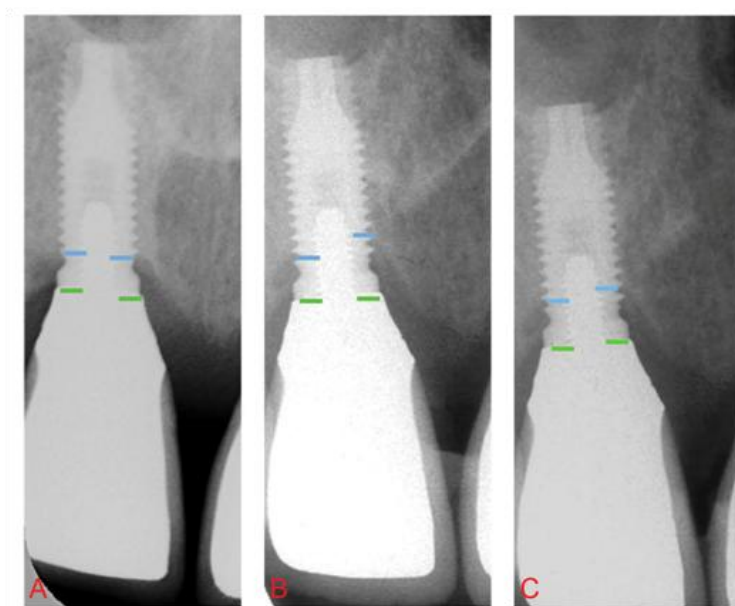


Figure 20.10. Radiographs of the same implant taken shortly after crown placement (A), one year (B) and ten years (C) post-loading. When (A) is used as the baseline image and compared with (C), marginal peri-implant bone loss can be clearly detected; this finding in association with BOP might lead to diagnosis of peri-implantitis. However, when (B) is used as the baseline image and compared with (C), a stable peri-implant bone level during a 9-year period can be clearly detected; this finding in association with BOP would lead to diagnosis of peri-implant mucositis.

For example, in a study evaluating prevalence of peri-implantitis after 9-14 years in function [104] a cut-off value of 3.1 mm for the distance between implant shoulder and first bone-to-implant contact was used for a case to qualify. Therefore, taking into consideration the possible early marginal peri-implant bone loss as a result of remodeling, it is inappropriate to use radiographs taken immediately after implant installation or crown delivery as the “baseline” radiograph when evaluating the long-term outcome of treatment; instead, radiographs taken about one year post-loading seem more appropriate for this purpose.

Conclusion

Panoramic images can be used for selecting the appropriate implant size and position during treatment planning in straightforward cases, provided that the distortion of the image is taken into consideration by a calibration procedure, and for obtaining an overview of the jaws and teeth. Panoramic images are not appropriate for research purposes for evaluating details regarding the outcome of treatment or for monitoring the peri-implant bone level where measuring precision is required, and should not be considered adequate for evaluating marginal peri-implant bone conditions in the clinic. 3D imaging, such as CBCT, may be used for treatment planning when information on the bucco-lingual dimension of the alveolar bone cannot be adequately obtained by a clinical examination before implant insertion. CBCT is however not suitable for follow-up examination due to metal beam hardening artifacts in the image sections. Intraoral periapical radiographs possess the best spatial resolution, and therefore sharp images can be obtained for assessment of treatment outcome and for monitoring of the implant over time. In periapical radiographs the marginal peri-implant bone level can be estimated with a relatively good precision, provided that sharp threads on both sides of the implant in threaded implants are displayed. This is achieved when the radiation beam is aiming perpendicular to the long axis of the implant and the film during exposure; a useful tool for eventually obtaining sharp threads in the image is the RB-RB/LB-LB rule as suggested. When evaluating the long-term outcome of treatment it is inappropriate to use radiographs taken immediately after implant installation or crown delivery as the “baseline” radiograph; instead, radiographs taken about one year post-loading should be used for this purpose.

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Chapter XXI

Osstell Measurements – The Use of Resonance Frequency Analysis (RFA) for Stability Assessment of Dental Implants

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Abstract

Achievement and maintenance of implant stability are prerequisites for the clinical success of osseointegrated dental implants. Resonance frequency analysis (RFA) is a technique for non-invasive assessment of implants stability. The technique has been developed in a multidisciplinary project to a commercially available instrument called Osstell. It makes use of a transducer that is attached to an implant, excited over a range of frequencies and where after the resultant resonance frequency (RF) of the transducer is measured.

The Osstell instrument uses calibrated transducers and gives measurements in Implant Stability Quotients (ISQ) from 1 ISQ (lowest stability) to 100 ISQ (highest stability). Experimental and clinical research during 15 years have shown the Osstell technique to provide clinically relevant information about the state of the implant–bone interface at any stage after implant placement.

The underlying RFA measurements translated into ISQ units evaluates implant stability in bending as a function of interface stiffness. Therefore, ISQ measurements reflect local bone density and are among several factors influenced by implant placement technique, healing time and exposed implant height above the alveolar crest. The technique can be used as one parameter in experimental and clinical research aimed to

evaluate new implant designs or treatment modalities. The Osstell technique can also be used in clinical routine as a diagnostic tool, since implants with high and increasing ISQ values represents successful implants, while low and/or falling readings may be a sign of ongoing failure.

21.1. Introduction

Dental implants represent one of the most successful treatment modalities in modern medicine. Nevertheless, failures do occur and according to systematic reviews about 5 to 8% of implants used in routine procedures [1,2] and up to 20% of implants placed in major grafting cases [3] are lost after 5 years and longer. Since low primary implant stability, low bone density, short implants and overload have been identified as risk factors, [1,4] the majority of implant losses may be explained as biomechanically induced failures. It can be assumed that these factors negatively influence the integration process during healing by inducing micro-mobility at the bone-implant interface, which in turn stimulates to fibrous tissue rather than bone formation. For successfully integrated implants, it can be speculated that an imbalance between stability and load creates a relative overload situation, which may lead to demineralization of the interface and loss of implant stability when loading is commenced. Thus, achievement and maintenance of implant stability are pre-conditions for a successful clinical outcome with dental implants. This suggests that techniques to measure and monitor implant stability are essential when developing and evaluating new implant designs and techniques in the research laboratory as well as in clinical trials. Such techniques would also give the clinician a possibility to optimize implant treatment, for instance by ensuring sufficient stability before loading and confirming maintained stability after a period of loading. A dental implant in clinical function is loaded in axial, lateral and rotational directions [5] (Figure 21-1).

The axial loads can be in intrusive or extrusive directions. Lateral loads can principally occur from any 360° direction around the implant. Rotational loading can be either clockwise or counter-clockwise. Thus, the outcome of an implant stability measurement depends on the type of test, the direction and magnitude of the applied force. [5] A clinically stable implant displays mobility on the micro-scale when loaded (Figure 21-2).

When applying a load to a clinically stable implant, the implant will be displaced in bone. [6] Thus, a stable implant can exhibit a varying degree of stability (i.e. different degrees of displacement or resistance to load). The main determinants of implant stability are the mechanical properties of the bone tissue at the implant site and how well the implant is engaged with that bone tissue. Thus, the bone density, the surgical technique and the implant design determine primary implant stability at the time of surgery. The implant is initially stabilized by lateral compression of bone (Figure 21-3). If the implant has a collar wider than its body, bone will also be compressed between the threads and the collar in an axial direction when the collar contacts the marginal bone. Secondary stability is achieved with time as a result of bone healing, i.e. bone resorption, bone formation and remodelling at the implant surface. New bone will bridge and fill the voids of the interface zone and grow into surface irregularities and macroscopic undercuts, which results in interlocking and further stabilization of the implant. The newly formed bone matures with time, which results in an increased density and stiffness of the implant-bone complex (Figure 21-4).

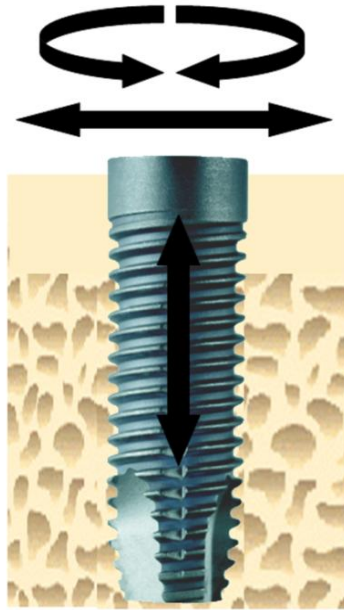


Figure 21.1. Schematic showing loading of a dental implant from different directions.

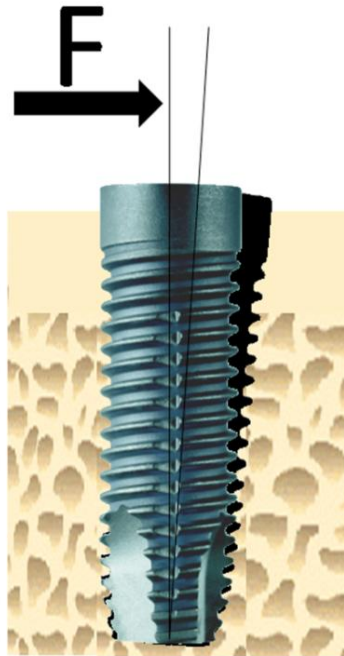


Figure 21.2. Showing lateral loading and displacement of an implant in bone.

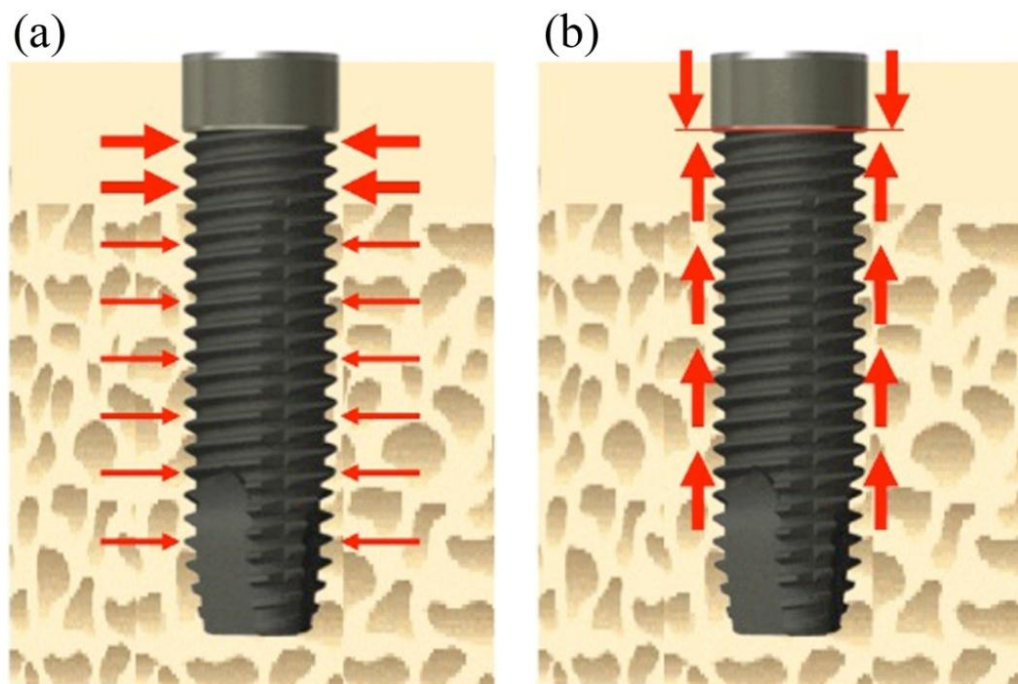


Figure 21.3. Showing primary implant stability due to (a) lateral and (b) axial compression of the bone at the implant site.

Destructive techniques such as push-out /pullout [7,8] and removal torque [9] tests have since long been used to evaluate the anchorage of implants in bone. It is generally accepted that the former measures a parameter related to the bone properties surrounding the implant rather than testing the implant-bone interface itself. [10] The removal torque test measures the strength of the interface in shear and is relevant when evaluating the rotational stability of an implant. Non-destructive methods such as PerioTest [11] and Resonance Frequency Analysis (RFA) [12] have been utilized for implant stability measurements *in vivo*.

The outcomes of measurements with both these techniques reflect the lateral stability of the implant by measuring contact time (PTV) or the resonance frequency (RF) of the system, respectively. [10] The RFA technique is a bending test of the implant–bone complex where the transducer applies an extremely small bending force and the RF reflects the displacement of the implant. [10] The RFA technique is regarded as more sensitive than the PerioTest one [5] and has been extensively used in experimental and clinical research for the last 15 years. The purpose of this chapter is to present the current knowledge about the resonance frequency analysis technique and to discuss the utility of the technique in dental implant research.

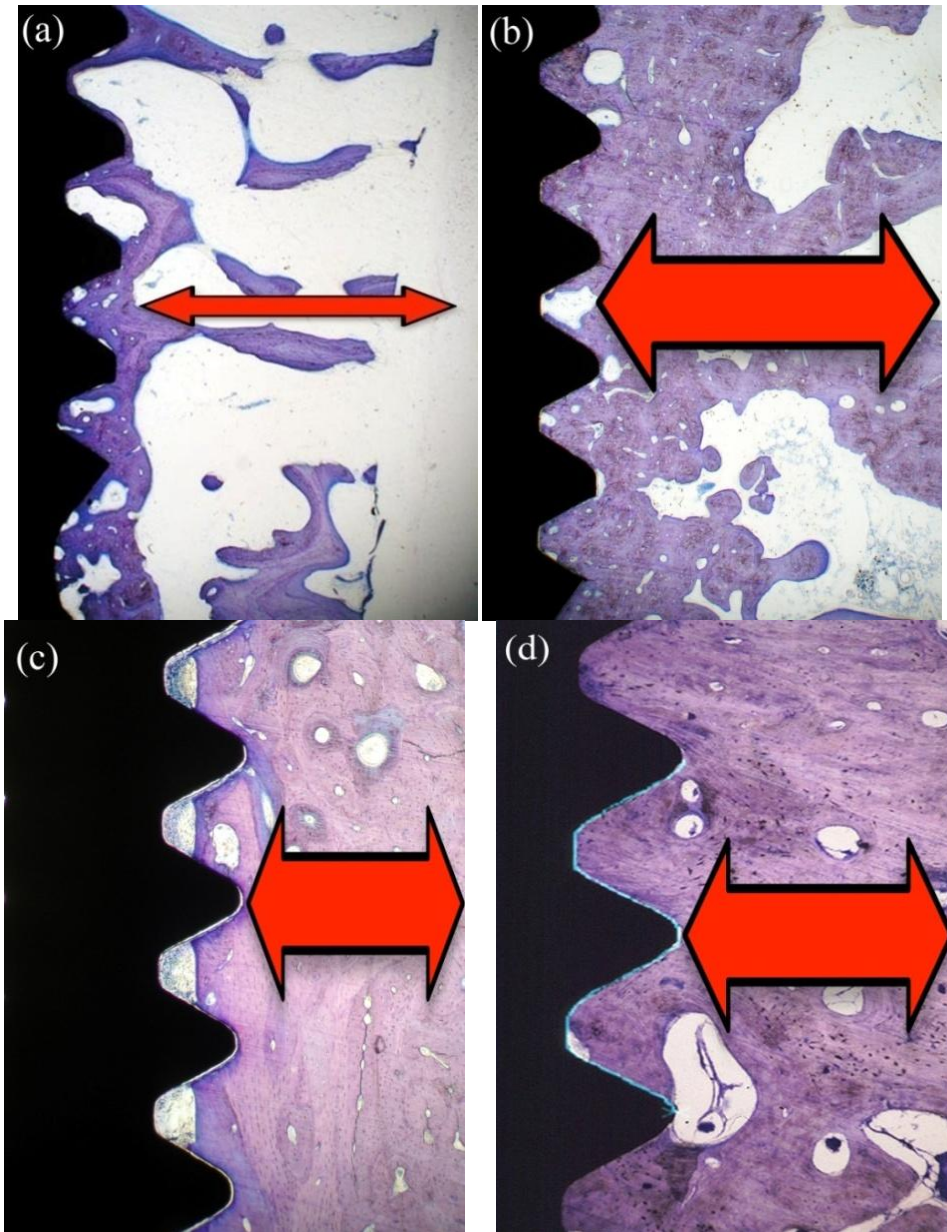


Figure 21.4. Light micrographs of ground sections showing implant placement and healing in different bone densities. (a) An implant in low density bone, i.e. slender bone trabeculae and soft bone marrow tissue. The arrow exemplifies low resistance to bending and low implant stability. (b) The same situation as (a) after some months of healing. Bone formation and remodelling has resulted in more BICs and increased peri-implant bone density. Arrow exemplifies increased resistance to bending as a consequence of increased bone density. (c) An implant in dense cortical bone. Implant stability is high. (d) Same situation as (c) after some months. Bone formation and remodelling has resulted in more BICs but no apparent changes of peri-implant bone density. Implant stability can be similar as after placement.

21.2. Resonance Frequency Analysis (RFA)

21.2.1. RFA Begins

After some years of work, Meredith and co-workers first described the RFA method in 1996 [12] where the first flexural resonance frequency of a transducer attached to an implant was measured *in vitro* and *in vivo*. The first generations of RFA utilized a transducer fabricated from stainless steel or titanium and comprised an offset cantilever beam. Piezoceramic elements had been attached to either face of the beam (Figure 21-5). The beam was vibrated by exciting one of the elements with a sinusoidal signal of varying frequency typically from 5 to 15 kHz, which was synthesised by a frequency response analyser and a PC. The second piezoceramic element measured the response of the beam and a charge amplifier amplified the signal generated. At the first flexural resonance frequency of the beam, there was a marked increase in amplitude and change in phase of the received signal. The RF at which the peak appeared was used to describe the stability of the implant in Hertz (Hz). From the early *in vitro* and *in vivo* work it was evident that the technique was sensitive to changes of interfacial stiffness (Figure 21-6) and influenced by the distance from the transducer to the first bone contact (Figure 21-7). [12].

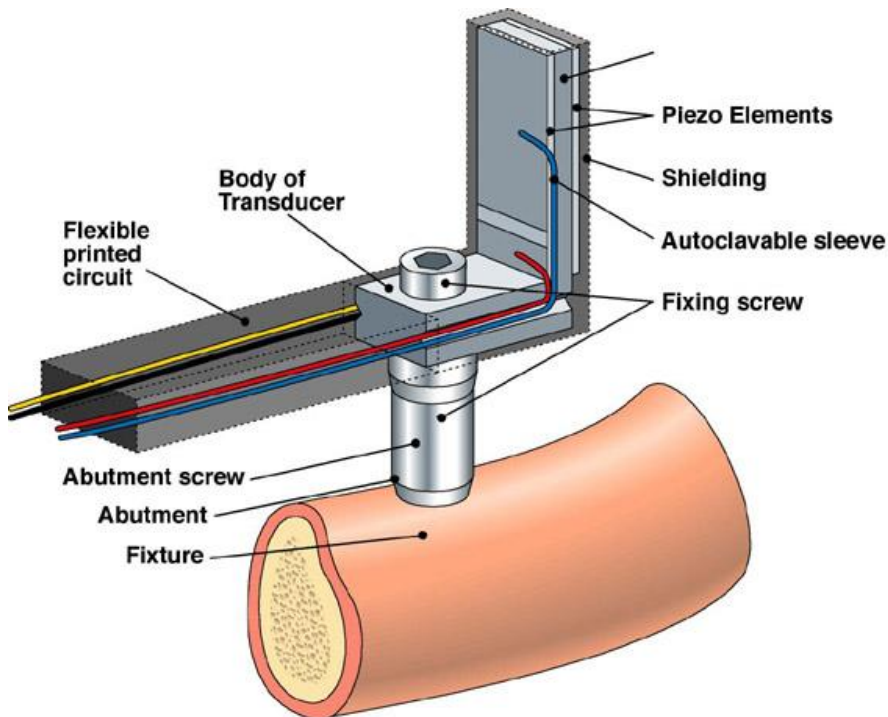


Figure 21.5. Schematic showing the design of the first generation of RFA transducers.

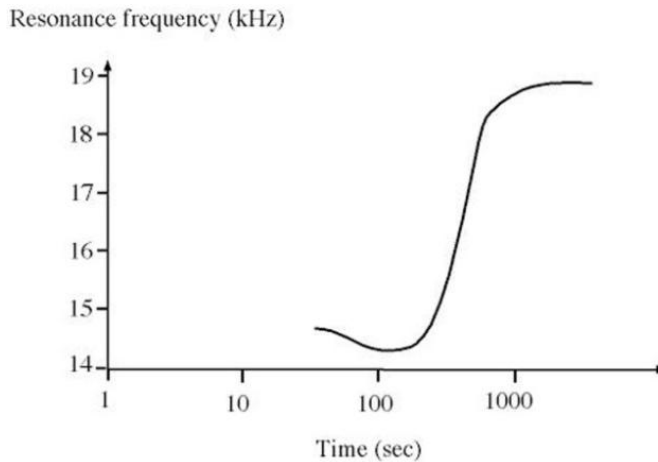


Figure 21.6. Showing the relation between RF and interface stiffness for an implant during setting in light curing resin.

It was also evident that implant stability was measured in the direction the transducer was fixed and that stability could be mapped 360 degrees around an implant. [13] Thus, the technique was suggested sensitive to detect variations of bone anatomy at an implant site and the presence of defects and marginal bone resorption.

The first generation of transducers had different fundamental RFs and the transducers had to be calibrated to achieve comparable results. Calibrations were made on one calibration block to give a nominal RF. The difference to this value was either subtracted or added to the measurements. The approach had obvious disadvantages since the first transducers tended to break and that several transducers had to be used in longitudinal studies. Another disadvantage was that the RF scale from approximately 3000 Hz to 9000 Hz was difficult to communicate. Moreover, the equipment was heavy and measurements were time consuming. Thus, further work was focused on developing a dedicated instrument, calibrated transducers and a new unit for stability measurements.

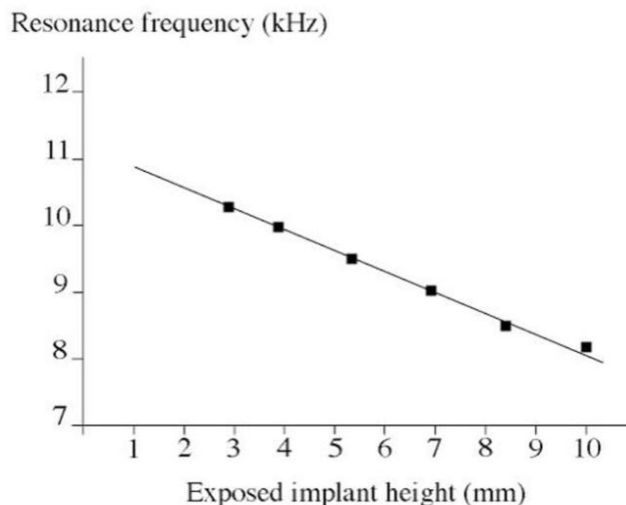


Figure 21.7. Showing the relation between RF and exposed implant height in an aluminium block.

21.2.2. Development of RFA as a Commercially Available Technique - Osstell

21.2.2.1. *Osstell, Calibrated Transducers and ISQ Units*

Thanks to a grant from the European Commission for a demonstration project between 1997 and 2000, [14] it became possible to develop a commercially available instrument. The most evident changes compared to the early prototypes were related to the electronics, the design and function of the transducer and a new measurement unit. Thus, a dedicated computer (Osstell, Osstell AB, Gothenburg, Sweden)) and autoclavable transducers could be fabricated (Figure 21-8). Moreover, a new unit called Implant Stability Quotient (ISQ) was established. The ISQ scale runs from 1 to 100 units, where the former is the lowest and the latter the highest degree of stability. The scale is close to a linear mapping of the underlying Hz-scale. ISQ is defined by a set of calibration blocks representing different stabilities. Transducers from the early clinical studies were used, when defining the relationship between ISQ and Hz for the first time. Five calibration blocks representing different stabilities were manufactured (Figure 21-9). The range of RF between the blocks was close to the range of previous RF measurements in the clinical studies. A linear relationship between resonance frequency and ISQ was used and the blocks with the lowest and highest stabilities defined the end points, i.e. 1 ISQ and 100 ISQ. The ISQ values for the three remaining blocks were then defined by simple mathematics. The five blocks were used for calibration of all future transducers and the calibration parameters programmed into the transducer plug using a microchip.

Mathematically, ISQ is defined by the following equation:

$$\text{ISQ} = \text{FR} * \text{FR} (u + v * L) + \text{FR} * (k + n * L) + p + m * L.$$

where

FR = Measured resonance frequency

u,v,k,n,p,m = Calibration factor -1 to +1, resolution 2/32768.

L = Abutment length (for an abutment transducer)

The six parameters u,v,k,n,m and p are all programmed into a microchip located in each transducer plug.

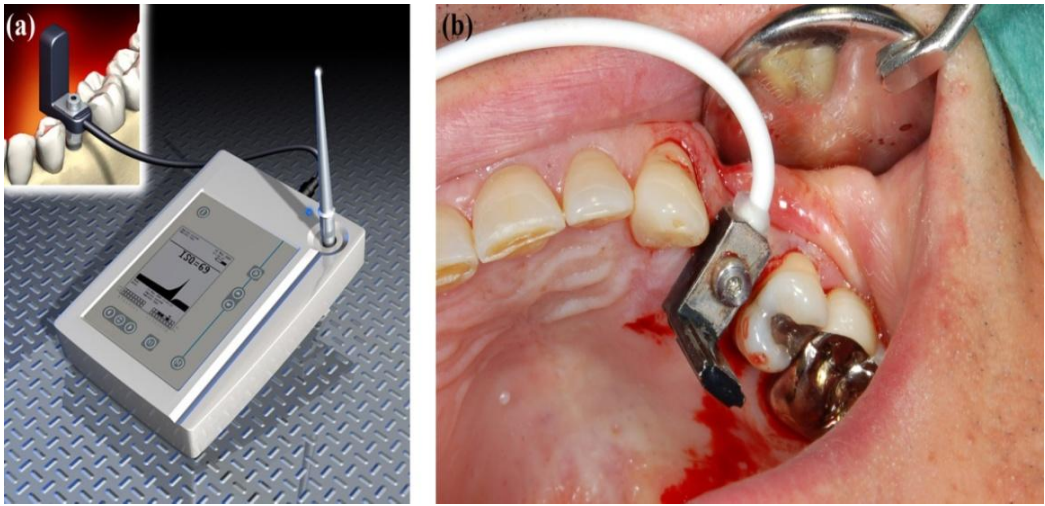


Figure 21.8. (a) The first Osstell instrument with transducer. (b) Clinical measurement with the Osstell transducer.



Figure 21.9. Calibration blocks of aluminium representing different ISQ values.

21.2.2.2. Wireless Osstell Measurements

With Osstell Mentor, the former wired transducer was replaced with a wireless aluminium rod with a magnet (SmartPeg), which allows for non-contact measurements (Figure 21-10). The RF of the peg is measured by the electronics by using the same principle as with the Osstell instrument.

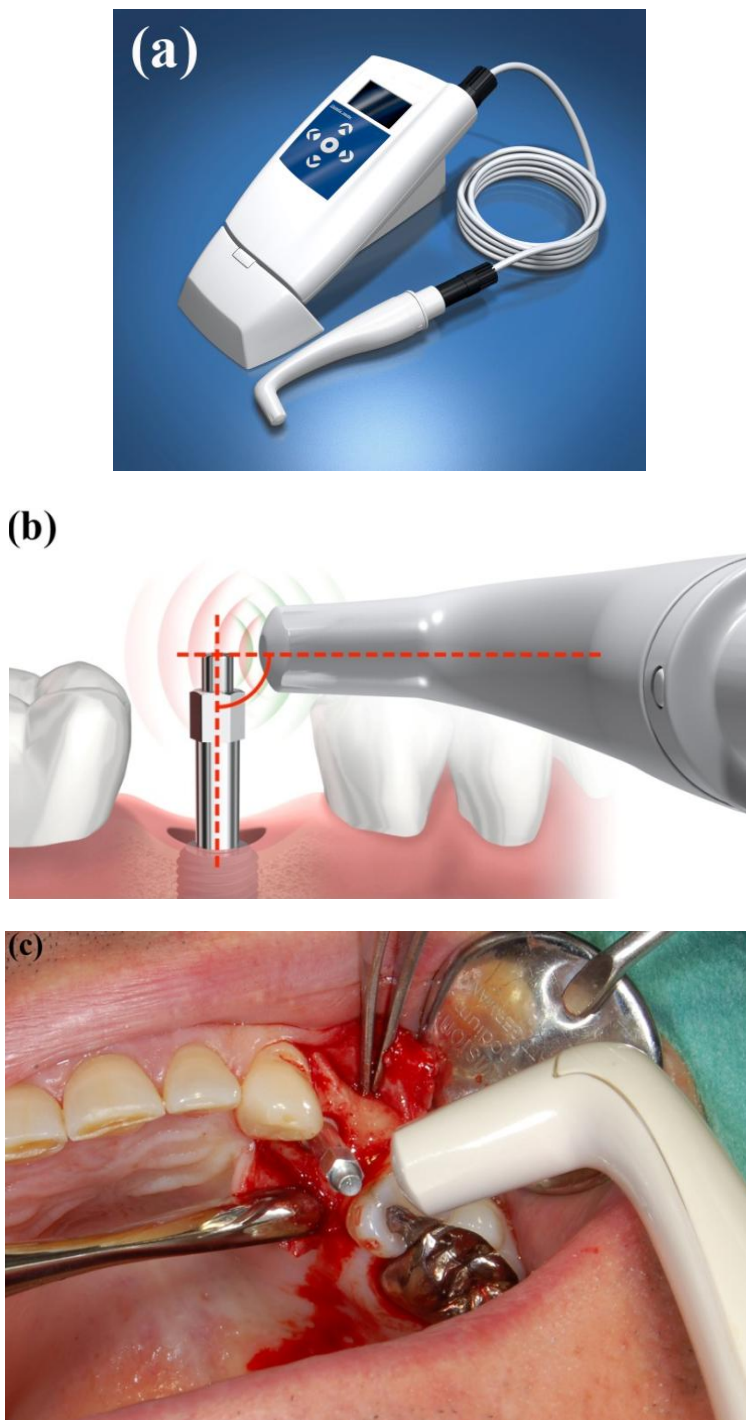


Figure 21.10. (a) The Osstell Mentor instrument and (b) SmartPeg transducer for wireless ISQ measurements. c/ Clinical measurements with the SmartPeg transducer.

However, while the transducer was excited with a swept sinusoidal signal through a cable, the magnet attached to a Smartpeg is excited with magnetic pulses. After each pulse, an electric coil in the measurement probe picks up the alternating magnetic field that is the result

of the self-vibrating Smartpeg. A second coil in the same probe generates the magnetic pulses.

The metal pegs have a simplified mechanical design compared to the transducers, and do not require individual calibration. It is not possible to store any calibration parameters in them since they are not electrically connected to the instrument. Instead, the individual differences between pegs are reduced to a minimum by a carefully controlled manufacturing process. The pegs also have a simpler mechanical behaviour when they are vibrating at their resonance frequency; they are more sensitive and have a predictable behaviour down to very low implant stability.

The first Osstell® Mentors were equipped with a 2-coil measurement probe. This version made use of three magnetic pulses in order to measure the response and calculate the ISQ from the two main resonance frequencies (corresponding to the lower and the higher ISQ). The 2-coil measurement probe was sensitive to surrounding electromagnetic noise, for instance from computers, TV-sets etc. Moreover, pulsing and ISQ calculation took a relative long time about 8 seconds. Later firmware versions use a new one-coil probe and four different pulses, which facilitates vibration of the SmartPeg, especially in cases with high damping by surrounding soft tissue. The ISQ calculation is much faster meaning that the time for a measurement cycle is much shorter (3 seconds). This probe is less sensitive to electromagnetic noise than the former version.

Osstell ISQ (Figure 21-11) is an improved version of Osstell Mentor. Osstell ISQ uses the same SmartPegs as Osstell Mentor but a measurement is made based on thirty pulses instead of four and cover the frequency spectrum from 1 to 10 kHz. Since the pulses are narrower than the pulses for the previous version, the thirty pulses contain more energy. This makes the responding signal stronger and the signal to noise ratio is improved, making Osstell ISQ even less sensitive to surrounding electromagnetic noise (Figure 21-12).



Figure 21.11. The latest Osstell ISQ instrument.

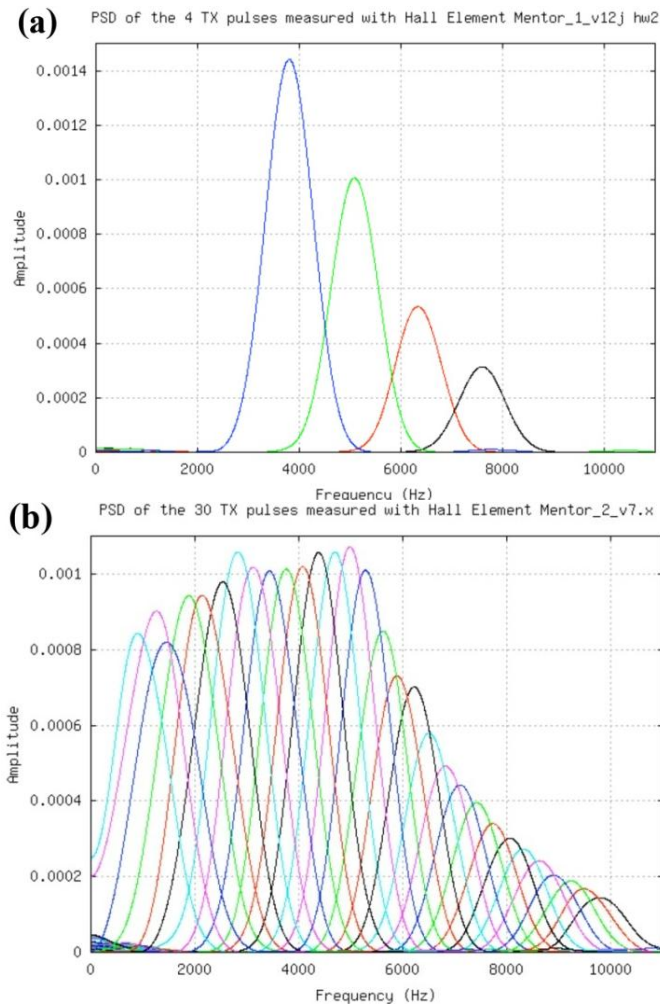


Figure 21.12. Magnetic pulse diagrams from (a) Mentor and (b) Osstell ISQ instruments.

21.2.2.3. Wired transducer vs Wireless Osstell Measurements

When comparing the Transducer-ISQ with the SmartPeg -SQ, the latter is more sensitive since it is possible to get an ISQ value down to 1, while the transducer is not working at such low stabilities. To make this possible, the ISQ will be slightly different with Mentor compared to Osstell® (Figure 21-13). With SmartPegs, the differences in ISQ between different implant systems have been minimized. For instance, a Straumann implant, which is placed with the 3 mm high collar above the bone, used to have lower ISQ compared to an implant placed in level with the bone. That difference is now adjusted for, which means that higher and correct ISQ values will be obtained with Osstell® Mentor compared to Osstell®.

The wired Osstell® transducer measures the stability in the direction in which it is mounted. Thus, measurements in different rotational directions will give different ISQ values (Figure 21-14).

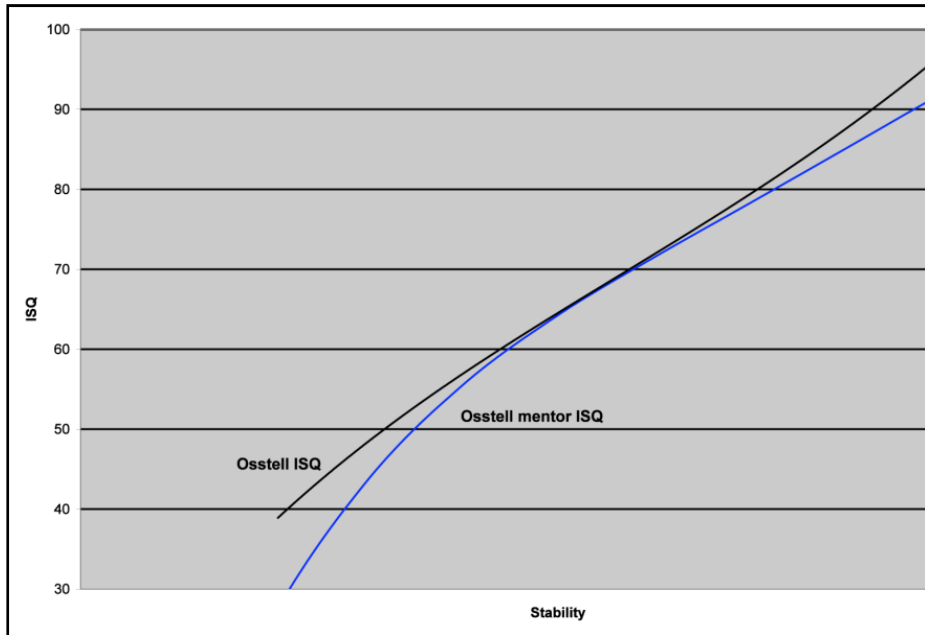


Figure 21.13. Graph describing the relationship between Mentor ISQ and Osstell® ISQ on a screw-shaped standard implant. The relationship has been determined with implants embedded in curing plaster.

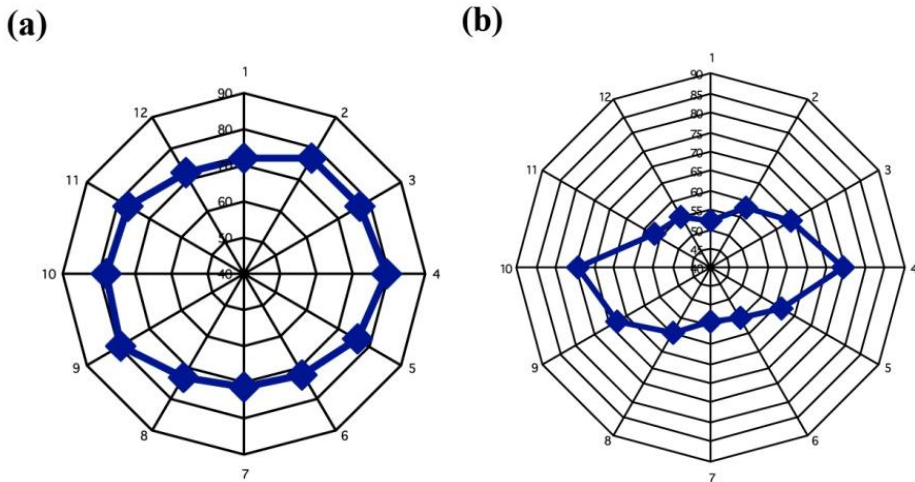


Figure 21.14. Graphs showing ISQ measurements 360° around implants placed a/ in thick bucco-palatal width and b/ in a site with thin width.

In most cases, the lowest stability is found in the bucco-lingual direction, which reflects thinner bone on this direction. In an extreme case, the difference between bucco-lingual and mesio-distal stability can be marked. Since it has been recommended to make measurements with the transducer in a buccal-lingual direction, it is possible that a false low value is measured, since the stability may be higher in mesial-distal direction. The SmartPeg is symmetrical and vibrates at two frequencies simultaneously.

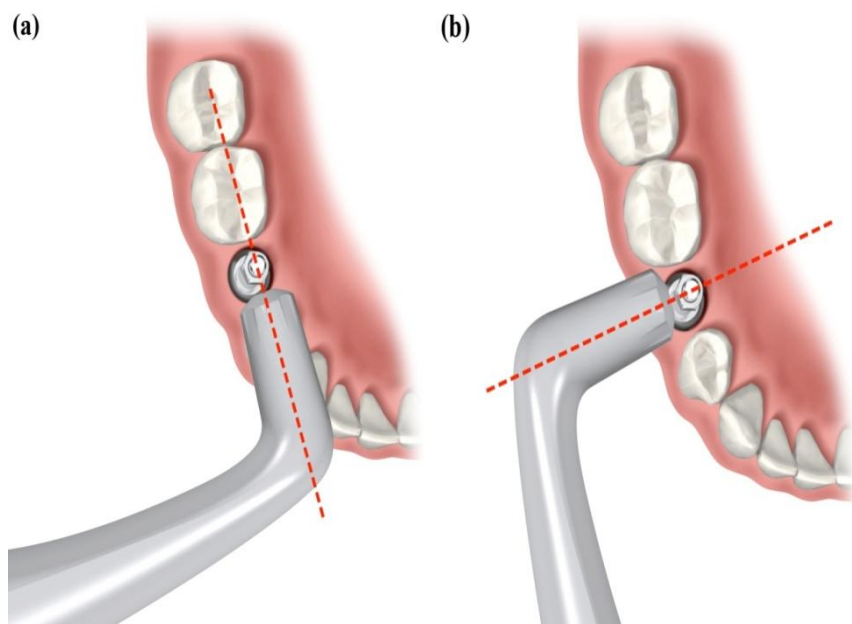


Figure 21.15. (a) and (b). Showing ISQ measurements from two directions perpendicular to each other.

These two frequencies correspond to the lowest and the highest stability direction for a specific implant (the directions are normally perpendicular to each other). This means that two values can be achieved on each implant (sometimes these values can be the same, for an implant with symmetrical stability). The lowest and highest stability directions correspond normally to the bucco-lingual and the mesiodistal direction, respectively. To ensure that two stabilities are detected, it is recommended to make two measurements on each implant, holding the probe from two different directions (Figure 21-15). For a totally symmetrical case, the values from the two instruments are comparable. However, most cases are not symmetrical, meaning that ISQ from Osstell® and Osstell® Mentor will differ. This is due to the direction-dependence of the Osstell® transducer, imperfections of the Osstell® transducer technique and the fact that the Osstell® transducer will not work at very low stabilities (<45 ISQ).

21.3. Factors Determining Osstell Measurements

21.3.1. Primary Implant Stability

21.3.1.1. Bone Density

Since RFA is sensitive to interfacial stiffness it makes sense that bone density has a major impact on Osstell measurements. Numerous studies have found a positive correlation between ISQ units and bone density as assessed with the Lekholm and Zarb index, [15-20] with insertion torque measurements [21-30] and with quantitative CT. [22, 24-25,28,31-32] Implant stability is usually higher in the mandible than in the maxilla, [16,33-35] since

maxillary bone is often softer than mandibular. It is also possible to find differences when comparing anterior and posterior sites within each jaw [3,34].

21.3.1.2. Marginal Cortical Bone Thickness

Studies using radiography and histology have indicated that the properties of the marginal bone influences Osstell measurements. [36-41] For instance, Miyamoto et al [37] observed a strong, positive correlation between cortical bone thickness, as judged from computed tomography scans and initial ISQ values for 225 screw-shaped implants placed in the maxilla and the mandible. Similarly, Nkenke et al [38] and Gedrange et al [39] found a positive correlation between the height of the crestal cortical bone and ISQ values in cadaver studies. In an *in vitro* study, and co-workers [40] used three screws to stabilize an implant at four different levels. The resonance frequency decreased when unscrewing the most coronal screws but not with the loss of the more apical screws, which suggests that the marginal region is the most important for the outcome of resonance frequency analysis measurements. In an *in vitro* investigation, Tözüm and co-workers [41] demonstrated a decreased ISQ value with decreasing bucco-lingual thickness from 8 to 0 mm.

21.3.1.3. Implant Length and Diameter

The findings regarding the influence of implant length and diameter on Osstell measurements are not conclusive. Östman et al [34] found higher stability with increased implant diameter but decreasing stability with increasing implant length. Miyamoto et al ³⁷ made a similar observation. This may be explained by the fact that some long implant designs have a reduced diameter (negative tolerance) in the coronal part to reduce friction heat and facilitate easy insertion. However, Sjöström and co-workers, [3] reported that the primary stability for the same implant design placed in grafted bone was significantly higher for 15 and 18 mm long implants than for 10 and 13 mm implants. Bischof et al [42] found no influence of implant position, implant length, implant diameter and vertical position on the ISQ values of 106 implants placed in the maxilla and the mandible, which is in line with the findings from other researchers [43-44]. Sim and Lang [19] reported a non-significant lower stability for 8 compared with 10 mm implants at placement, but that the 8 mm showed a significant increase up to 12 weeks. A clinical study found a higher stability for 12 than for 10 mm implants and for 4.8 mm than for 4.1 mm wide implants. [20] Also Tözüm and co-workers found higher ISQ values with increased implant diameter in an *in vitro* study [41].

21.3.1.4. Surgical Technique

The use of technique to create increased lateral compression during insertion seems to result in higher stability. This may be due to undersized preparation before placing the implant, [45] wider implants [46] or the use of tapered implant [47-48].

21.3.1.5. Gender

Clinical studies have demonstrated higher mean ISQ values in men than in females. [20, 23, 34, 49]. The reason is not well understood but may be related to the higher incidence of osteoporosis in women.

21.3.1.6. Orientation of the Resonance Frequency Analysis Transducer

As discussed earlier, the orientation of the wired transducer influences the resonance frequency analysis measurements. Authors have found that ISQ values increased by approximately 10 units when performing measurements with the wired transducer parallel compared with perpendicular to the alveolar crest. [50-51] This can be explained by directional differences in the local anatomy, i.e. it is likely that a thin buccal bone results in a lower ISQ value than dense bone in mesial-distal direction. [41] However, the new wireless resonance frequency analysis technique (MentorTM; Osstell AB) can detect such differences if exceeding 3 ISQ units. Valderrama et al [52] found that the two RFA techniques can differ by up to 10 ISQ units, with higher stability values obtained in the mesial-distal direction with the wireless technique and lower values obtained in the buccal-palatal direction with the old technique.

21.3.2. Secondary Stability

21.3.2.1. Time Dependence

The resonance frequency analysis technique has been used in animals to study implant healing in normal bone, [53-54] in grafted bone [55-57] and in membrane-induced bone. [58] In the rabbit tibia model the resonance frequency increases with time as a function of an increased stiffness resulting from new bone formation and remodelling. However, if the primary stability of an implant is very high, as can be achieved in the dog mandible, subtle changes in stiffness may not be evident [59-61].

Friberg and co-workers [21] reported that all implants placed in the edentulous maxilla, irrespective of initial stability, tended to reach a similar level of stability at the time of abutment connection (6– 8 months later) and after 1 year in function. This is in line with a clinical study by Sennerby et al, [50] where implants in soft bone with low primary stability showed a marked increase in stability compared with implants in dense bone. Other researchers, [3,16,33,62-64] have reported similar findings. The data indicate that healing and remodelling process of soft trabecular bone seems to result in an increased stiffness of the peri-implant bone.

Studies on one-stage and immediately loaded implants have demonstrated an initial decrease of implant stability, which, however, seems to reverse after 3 months when an increase in implant stability is usually seen. [16, 65-68]. The initial decrease in implant stability is probably reflects the healing and remodelling process and thereby a temporary weakening of the bone. It can be speculated that loading of the implants during this period may accentuate this initial decrease of ISQ value. [65] However, studies have also shown no initial dip, [69-71] which may be explained by that different implant surface has been used.

21.3.2.2. Marginal BONE RESORPTION and PResence of DEFECTS

The relationship between the length of an implant abutment and resonance frequency analysis data has been examined in various model systems. Ohta and co-workers [29] demonstrated a correlation between ISQ readings and the size of 0.5 mm deep peri-implant defects.

Turkyilmaz et al [72] demonstrated a negative correlation between exposed implant height and ISQ values for implants placed in fresh extraction sockets in human jaws. The authors proposed using the resonance frequency analysis technique to monitor the healing of implants in extraction sockets. Similar results were reported by other researchers [73-74].

Meredith et al [12] measured the frequency response of the transducer attached to an implant fixture in an aluminium block using abutments of various lengths. In a dog study on peri-implant breakdown, in a peri-implantitis dog model, Sennerby et al [60] demonstrated a negative correlation between radiographic bone loss and ISQ measurements. It should be noted that marginal breakdown was initiated after healing and integration of the implants. Meredith et al [75] studied 52 maxillary implants after at least 5 years in function and revealed a significant, positive relationship between effective implant length (abutment length + bone loss) and resonance frequency. The study implants showed a similar degree of stability after 5 years of function. In a study on one-stage implants in dense mandibular bone, a small, but significant, decrease in stability was detected over a 15-week period, which was probably caused by marginal bone loss and an increased exposure of the implant above the bone crest. [76].

Turkyilmaz and co-workers [77] found a negative correlation between increased marginal bone loss around mandibular implants and decreased implant stability over the first 6 months following implant placement. No such correlation was observed between the 6-month and the 12-month study period. The authors suggested that the effect of bone loss was compensated for by an increased interfacial stiffness resulting from bone formation and remodelling. In a clinical study on mandibular implants, Tözüm et al [78] noted a negative correlation between ISQ and marginal bone resorption. However, Fischer et al [69,79] found no correlation between marginal bone loss and resonance frequency analysis measurements during a 1-year period. The ongoing healing process may have counteracted and masked the effect of marginal bone loss.

However, after 3 and 5 years, when healing must be regarded as being complete, the same research group found a strong, positive correlation between marginal bone resorption and low ISQ values. This is in line with Meredith et al, [75] who suggested that variations in implant stability after 5 years in function could be explained by differences in marginal bone height.

21.3.2.3. Implant Surface

Most researchers have not found implant surfaces to impact on implant stability. [60, 80-82] However, in dogs, Rompen et al [59] showed that surfaced-modified implants maintained stability, whilst machined implants experienced a decrease in stability during the early healing period.

This has been confirmed in two clinical studies. Glauser et al [83] compared machined and oxidized implants using an immediate loading protocol and found more decrease in stability for machined implants during the first 3 months post-loading. A clinical study on immediate loading in the posterior mandible found no difference in primary stability between machined and oxidized titanium implants. [84] However, the machined implants showed a significant loss of stability, while the oxidized implants remained their stability after 4 months of loading.

21.4. Correlations with Other Techniques

21.4.1. Histology and Histomorphometry

Most studies have failed to show a correlation between the degree of implant–bone contacts (BICs) and resonance frequency analysis measurements. [53,59-61,84-86] For some colleagues this may seem as surprising, since there is a perception that percentage of BIC is strongly associated with osseointegration. [61] However, osseointegration is a biomechanical concept where BICs has a role as a consequence of bone healing at an implant surface. However, the degree of BIC does not necessarily reflect the stiffness of the surrounding bone. For instance, rough surfaced implants is often covered by a thin layer of bone, which with morphometry results in high percentage of BIC but is not important for the biomechanical support of implants. Most studies have not found the type of implant surface to have an impact on implant stability [60, 80-82].

21.4.2. Insertion Torque Measurements

As previously discussed most studies there is a clear correlation between mean insertion torque or peak insertion torque and ISQ measurements. [21-30]. This is explained by the fact that insertion torque in turn correlates with bone density.

21.4.3. Bone Density Measurements in Radiographs

Clinical and experimental studies have shown correlation between ISQ and bone density as measured in Hounsfield units (HUs) in CT scans [22, 24-25, 28, 31-32] or with fractal dimension measurements in panoramic radiographs [87].

21.4.4. Displacement Measurements

In an *in vitro* study, implants were placed in different bone densities and with different insertion torques. [88] A lateral load of 25 N was applied to each implant and the displacement measured. A high dependence between ISQ values and displacement was reported.

21.5. ISQ and Failing Implants

From a clinical point of view, it seems logical to assume that implants with low and/or falling ISQ values may be indicative of ongoing implant failure. In a study by Friberg et al [76, 75] one-stage implants in the edentulous mandible was evaluated with RFA. One implant showed a decreasing stability from week 2 to week 15, when the implant was found to be clinically mobile. In a second patient, three of five implants showed a marked decrease in

stability from week 2 to week 6, which corresponded to the period of implant loading with a relined denture. After a period of unloading, the implant stability increased for two implants and was maintained at the same level for one implant. The same research group showed an increase in implant stability from the time of placement to abutment connection for 56 maxillary implants except for two failing implants. [21] In an immediate loading study, Glauser et al [66] monitored the stability of 81 implants from placement to 1 year in function. A total of nine implants failures were experienced. All implants showed a high degree of initial stability, around ISQ 70, but the group of future failures showed a continuous decrease in implant stability. After 1 month, the mean ISQ value of 52 was statistically lower for the group of future failures than for the successful implants, which showed an ISQ of 68. Also, ISQ values of 49–58 were associated with an 18.2% risk of failure. Evidently, the lower the ISQ value after 1 month of immediate loading, the higher the risk for future failure.

In a follow-up study on implants placed in extraction sockets and subjected to immediate/early loading, Vanden Boagerde and co-workers [89] demonstrated rescue of one implant based on resonance frequency analysis measurements. This implant showed a significant drop from 67 ISQ to 53 ISQ during the first six weeks. The implant was unloaded and recovered to an ISQ value of 72 after 6 months.

Sjöström et al [3] found lower primary stability for 17 implants (ISQ 54.6) that failed during the first year of function compared with 195 implants (ISQ 62.0) that were successful installed in grafted maxillae.

Nedir and co-workers [62] compared immediately loaded implants with implants loaded after 3 months of healing and concluded that the resonance frequency analysis technique did not reliably identify mobile implants. However, implant stability could be reliably determined for implants with an ISQ of more than 47. One explanation for not detecting some mobile implants may be a result of the nature of the resonance frequency analysis technique, which measures stability as a function of stiffness. Clinically mobile implants display an exceptionally low stiffness, which prevents the resonance frequency analysis system from identifying the first resonance frequency, and which therefore records a falsely high ISQ value corresponding to the second resonance frequency [90].

Huwiler et al [91] followed 17 implants with repeated resonance frequency analysis measurements for up to 12 weeks after implant surgery (24). One implant failed and its ISQ value decreased from 68 to 45. As implant mobility occurred at low ISQ values, the authors concluded that the resonance frequency analysis system couldn't be used to predict implant failure.

Fischer et al [69] studied the stability of 53 implants during a period of 1 year (15). The implants supported single crowns ($n = 16$) or partial bridges ($n = 16$) in the maxilla placed at the time of, or within 16 days of, implant surgery. The average primary stability of all implants after surgery was 63.3 ISQ, and one failed implant showed a value of 56 ISQ, which was the fifth lowest value of the 53 implants. In another study, the same group performed resonance frequency analysis measurements in 24 patients with 139 maxillary implants at 3 and 5 years following implant surgery [79]. Four implants were lost during the third to the fifth year. At year 3, the failing implants showed lower ISQ values than the average implant (i.e. 44 ISQ, 53 ISQ, 54 ISQ and 54 ISQ vs. an average of 57.7 ISQ for all other implants in the study). An assessment of the risk for implant failure showed that ISQ values below 44, 53 and 54 were associated with failure rates of 100%, 6.7% and 9.5%, respectively. None of 97 implants with ISQ values higher than 54 failed from study year 3 to study year 5.

In a retrospective study on 300 implants of which 20 were lost after three years, Turkyilmaz and McGlumphy [25] found a significant difference between the failed and the successful implants with regard to primary stability and bone density. Thus, the failed implants showed lower ISQ values than the successful ones, 46.5 ± 4 vs 67.1 ± 7 ISQ. Similar significant differences were found for HUs and insertion torque.

In a clinical study comprising ISQ measurements of 542 implants, Rodrigo et al [92] experienced loss of 37 implants over a three-year period. They found no correlation between ISQ measurements of primary stability and implant failure but a significant association between measurements after a mean period of 2.8 months and implant failure.

Conclusion

The Osstell technique has the potential to provide clinically relevant information about the state of the implant–bone interface at any stage after implant placement. The underlying RFA measurements translated into ISQ units evaluates implant stability in bending as a function of interface stiffness. Therefore ISQ measurements reflect local bone density and are among several factors influenced by implant placement technique, healing time and exposed implant height above the alveolar crest. The technique can be used as one parameter in experimental and clinical research aimed to evaluate new implant designs or treatment modalities. The Osstell technique can also be used in clinical routine as a diagnostic tool, since implants with high and increasing ISQ values represents successful implants, while low and/or falling readings may be a sign of ongoing failure.

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Appendix 1. Abbreviations

ADME	absorption, distribution, metabolism and elimination
ALP	alkaline phosphatase
AES	<i>Auger Electron Microscopy</i>
BIC	bone-implant contact
BoP	bleeding on probing
BMP-2	bone morphogenetic protein-2
BMD	bone mineral density
BMP	bone morphogenetic protein
BSE	backscattered electron microscopy
CAL	clinical attachment level
CATK	cathepsin K
CLSM	confocal laser scanning microscopy
CT	computed tomography
CBCT	cone beam computed tomography
COL1A1	collagen type I alpha 1
CONSORT	consolidated standards of reporting trials
DBM	demineralized bone matrix
Dkk1	Dickkopf-1
E	elastic modulus (Young's modulus)
ECM	extracellular matrix
EDS or EDX	energy dispersive spectroscopy / X-ray analysis
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assays
EMD	enamel matrix derivative
F	force
FA	focal adhesion
FAK	focal adhesion kinase
FDA	the US Food and Drug Administration
FEA	finite element analysis
FIB	<i>focused ion beam</i>

FGF	fibroblast growth factor
GBR	guide bone regeneration
GCF	gingival crevicular fluid
GCP	Guidelines for good Clinical Practice
GFs	growth factors
GTR	guided tissue regeneration
HA	hydroxyapatite
hMSCs	human mesenchymal stem cells
hFOBs	human fetal osteoblasts
HZ	Hertz
ICH	international conference on harmonisation guidelines
IGF	insulin-like growth factor
IHCS	immunohistochemical staining
IL	interleukin
IM	intramuscularly
ISQ	implant stability quotient
ITI	international Team for Implantology
MAPK	mitogen-activated protein kinase
MMA	methyl methacrylate
MPa	Megapascals
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSC	mesenchymal stem cell
MSCT	Multi-Slice CT system
N	newton
Ncm	newton centimeter
Nm	newton meter
NPE	neutral proteolytic enzyme
OC	osteocalcin
OCT	Optical coherence topography
ON	osteonecrosis
OPG	Osteoprotegerin
OSX	Osterix
OVX	Ovariectomy
Pa	Pascals
PDGF	parathyroid hormone-related protein
PD	pharmacodynamic
PK	pharmacokinetic
PBPK	physiologically-based pharmacokinetic
PTHrP	platelet-derived growth factor
PD	probing pocket depth
PDL	periodontal ligament

PG	proteoglycan
PICF	peri-implant crevicular fluid
PMMA	polymethylmethacrylate
PMNs	polymorphonuclear leukocytes
qPCR	quantitative Polymerase Chain Reaction
RCT	randomized controlled trials
R_a	surface roughness parameter, arithmetic average
R_q	surface roughness parameter, root mean square
RF	resonance frequency
RFA	resonance Frequency Analysis
rRNA	ribosomal RNA
SBF	simulated body fluid
SEM	scanning electron microscopy
SLA	sandblasted, large-grit, acid-etched
Ti	titanium
TPS	Titanium plasma spraying
TNF- α	tumor necrosis factor- α
TGF- β	transforming growth factor- β
TEM	transmission electron microscopy
TD	toxicodynamic
β -TCP	β -tricalcium phosphate
XPS	<i>X-ray photoelectron spectroscopy (XPS)</i>
XRD	X-ray diffraction
XRF	X-ray fluorescence spectroscopy
σ	stress
E	strain

Appendix 2. Terms and Definitions

Basic research is research carried out to increase understanding of fundamental principles.

Many times the end results have no direct or immediate commercial benefits.

Bioactivity Spontaneous communication of a material in a biological environment resulting in strong adhesion between tissue and the material. Implant bioactivity may depend upon material composition, topography, and chemical or physical surface variations.

Biocompatibility A term used to describe the acceptance of a material as a biomaterial, that is, the material does not provoke rejection by the surrounding tissues and body as a whole. It is also the ability of a material to perform with an appropriate host response in a specific application.

Bioinert Describes a biomaterials that does not elicit a biologic response or is unaffected by the adjacent biologic environment.

Biomaterial is any matter, surface, or construct that interacts with biological systems. It used to replace a part or a function of the body in a safe, reliable, economic, and physiologically acceptable manner.

Biomechanics is the application of mechanical principles to biological systems, such as humans, animals, organs, and cells.

Clinical research A research study using consenting human subjects that tests the effectiveness and safety of a treatment, a diagnostic tool, or a prophylactic intervention.

Crevicular fluid Serum ultrafiltrate tissue fluid that seeps into the gingival sulcus from gingival connective tissue and vasculature through thin sulcular epithelia.

Finite element analysis (FEA) Science of creating computer simulations of mechanical or clinical situations.

Guided bone regeneration (GBR) are dental surgical procedures that utilize barrier membranes to direct the growth of new bone tissue at sites having insufficient volumes or dimensions of bone for proper function, esthetics or prosthetic restoration.

Histomorphometry a method used to accurately quantify the level of cellular activity and structure of a tissue (as bone) especially by computer-assisted analysis of images formed by a microscope.

Implant Medical device made from one or more biomaterials placed within the body, either totally or partially buried beneath an epithelial surface.

Implant dentistry is science and art concerned with treatment planning, reconstruction, and maintenance the health of the patient through replacement of teeth and contiguous structures with natural and alloplastic devices and materials.

Mini-implant Implant fabricated of same biocompatible materials as other implants but of smaller dimensions.

Osseointegration Direct structural and functional connection between living bone and the surface of a load-bearing artificial implant. (Original definition attributed to P-I Brånemark published in 1985 based on microscopic finding).

Osteotomy surgical removal of all or part of a bone using drill or chisels.

Osteoconductivity The ability of a structural scaffold to guide bone ingrowth.

Osteogenesis Generation, regeneration, and remodeling of bone via concerted action of bone cells.

Osteoinduction The process of stimulating osteogenesis. It involves the stimulation of osteoprogenitor cells to differentiate into osteoblasts that then begin new bone formation.

Translational research It is transforms scientific discoveries arising from laboratory, clinical or population studies into clinical or population-based applications to improve health by reducing disease incidence, morbidity and mortality.

Appendix 3. World–Wide-Web Sites

Societies and Professional Organizations

www.osseo.org	Academy of Osseointegration (AO)
www.aaid-implant.org	American Academy of <i>Implant Dentistry</i>
www.asid.org.au	Australian Society of Implant Dentistry (ASID)
www.camlogfoundation.org	Camlog Foundation
www.biomaterials.ca	Canadian Biomaterials Society
www.eao.org	European Association for <i>Osseointegration</i> (EAO)
www.iadr.com	International Association for Dental Research.
www.iti.org	International Team for Implantology (ITI)
www.icoi.org	<i>International Congress</i>
www.osteology.org	Osteology Foundation
www.scsb.eu	Scandinavian <i>Society for Biomaterials</i>
www.biomaterials.org	Society for Biomaterials
www.esbiomaterials.eu	The European Society for Biomaterials
www.bsoi.org	British <i>Society of Oral Implantology</i>
www.asid.org.au	<i>Australian Society of Implant Dentistry</i>
www.iaoci.com	International Academy of Ceramic Implantology
www.idia.org	Institute for Dental Implant Awareness

Research Institutes, Organizations

www.biomaterials.gu.se	Department of Biomaterials, University of Gothenburg
www.biomatcell.se	BIOMATCELL VINN Excellence Center of Biomaterials and Cell Therapy
www.nyu.edu/gsas/program/biomaterials	Department of Biomaterials and Biomimetics, New York University
www.mah.se/od/eng	Faculty of Odontology, Malmö University
www.georgiahealth.edu/dentalmedicine/Periodontics/lapcr	Laboratory for Applied Periodontal and Craniofacial Regeneration, Georgia Health Sciences University

www.lj.se/oi	The Institute for Postgraduate Dental Education Jönköping
www.griffith.edu.au/health/school-dentistry-oral-health	School of Dentistry and Oral Health Griffith University
www.acta.nl/en/	Academic Centre for Dentistry Amsterdam
www.odontologi.au.dk/en	School of Dentistry, Aarhus University
www.osstell.com	Osstell AB
www.med.utu.fi/dent/en/departmen/biomaterials	Department of Biomaterials, Institute of Dentistry, University of Turku
www.med.utu.fi/dent/en/departmen/prosthetic	Department of Prosthetic Dentistry, Institute of Dentistry, University of Turku
www.biomaterials.utu.fi	Turku Clinical Biomaterials Centre - TCBC
www.dent.ksu.edu.sa/chairs	Dental Implant and Osseointegration Research Chair, College of Dentistry, King Saud University
www3.chubu.ac.jp/life_health/english/departments/medicine	Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University
www.branemark.com	Brånemark Osseointegration Center
www.dentistry.unc.edu/depts/pros	Department of Prosthodontics, University of North Carolina
www.uni-mainz.de	Department of Oral and Maxillofacial – Plastic Surgery, University of Mainz
www.frame.org.uk	Fund for the Replacement of Animals in Medical Experiments (FRAME)
www.lagiannuzzi.com	L.A. Giannuzzi and Associates LLC
www.smd.unige.ch	School of dental medicine, University of Geneva
www.utoronto.ca/dentistry/facultyresearch	Dental Research Institute, Faculty of Dentistry, University of Toronto
www.dentistry.ubc.ca	Faculty of Dentistry at University of British Columbia

Dental Implant Companies

www.straumann.com	Institute Straumann AG
www.astratech.us	Astra Tech
www.bicon.com	Bicon Dental Implant
www.biohorizons.com	BioHorizons Implant Systems Incorporated
www.biomet3i.com	Biomet 3i Implant Innovations Incorporated
www.camlogimplants.com	Camlog Group
www.dentsply-friadent.com	Dentsply Friadent Ceramed Incorporated
www.nobelbiocare.com	Nobel Biocare
www.zimmerdental.com	Zimmer Dental
www.keystonedental.com	Keystone Dental

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